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Supplements

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factors" has been coined previously to identify hypothetical stimuli induced specifically by muscular activity that are active for temperature regulation If such stimuli exist, independent of the effect of increased metabolic rate and heat production, perhaps they could be elucidated by comparing the effect on temperature regulation of intermittent work, *i e*, a greater work intensity for a shorter time, but at the same average heat production as in continuous work It is well-known from earlier work in this laboratory that the two work procedures may produce different circulatory responses and different respiratory responses (Åstrand *et al* 1960 a) The purpose of the present study was, therfore, to investigate body temperature changes and heat dissipation responses to moderately heavy continuous and intermittent work at the same total heat production

Procedure and Methods

The three well trained male subjects were accustomed to the experimental procedures Preliminary maximal O_2 uptake tests (max $\dot{V}_{O_2}$) were done to help determine the proper workloads to be used (Table I) The subjects slept in the laboratory the night before their temperature experiments No food was allowed after the evening meal and no water was consumed prior to or during the experiment Each subject then worked at e her cont [illegible]
for 1 hr at a workloac [illegible]
$\dot{V}_{O_2}$ (rel $\dot{V}_{O_2}$) of 60 ° [illegible]
d r ng exerc se can [illegible]

[illegible]

least 5 min Expired gas was collected in Douglas bags the volume was measured in a 150 *l* Tissot tank (±0 05 *l* and the O_2 and CO_2 composition determined by a modified Haldane technique (Åstrand and Saltin 1961 The standard deviation of the differences between 11 duplicate max $\dot{V}_{O_2}$ determinations was 0 05 *l* [illegible]
mined from fingertip sam [illegible]
les were taken 2 to 4 min [illegible]
Continuous and intermitte [illegible]
and T_{re} were taken The [illegible]
upright position for 1 hr at either continuous or intermittent exercise (30 sec exercise and 30 sec rest During intermittent work the 2 min expired gas samples from the exercise and rest periods O_2 debt were collected and analyzed separately and the $\dot{V}_{O_2}$ values were averaged Oxygen debt was not measured after continuous work Blood lactates were taken periodically and the standard deviation of the method calculated from 25 duplicate determinations was for concentrations between 0 t 5 6 meq *l* → 0 23 (8 %, 5 6 to 11 1 meq *l* → −0 33 (6 %) and over 11 1 meq *l* → 0 43 4 %, The mean S D of four duplicate determinations on a single sample was 1 84 0 31 meq *l* Heart rates were measured from a continuous EKG record and during intermittent work

TABLE I Anthropometric and physiologic base line data on the subjects The highest maximal oxygen uptake values obtained from either the maximal or supramaximal tests are given

Subj	Age yr	Ht	Wt kg	DuBois S A m²	Max $\dot{V}_{O_2}$ STPD ml kg min	Max $\dot{V}_E$ BTPS l min	Max (f) br/min	Max $\dot{V}_E$ (f) l br	Max R_E	Max energy kcal (m² hr)
BEN	22	1 9	43	1 94	59 1	147 1	48 5	3 03	1 13	693 8
BON	22	1 8	37	1 82	65 1	160 1	57 4	3 00	1 26	673 8
STN	24	17	2	1 81	60 1	165 1	62 3	2 81	1 08	743 7
Mean	23	175 8	47	1 86	61 4	157 4	56 1	2 95	1 16	705 4

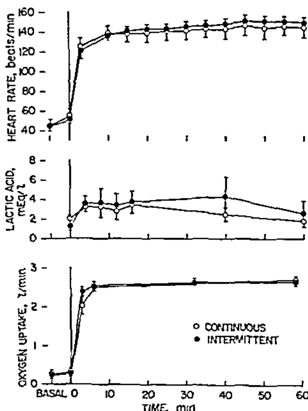


Fig 1 Individual oxygen uptakes heart rates and blood lactic acid concentrations during submaximal and maximal exercise

$$\text{Efficiency} = \frac{\text{mech work performed (kpm/min)} \times 100}{426\ 85\ \text{kpm/kcal (total kcal prod /min—basal kcal prod./min)}}$$

Total H_2O loss = [total wt loss − (118 614 $\dot{V}_{CO_2}$ − 85 742 $\dot{V}_{O_2}$)] × l/m^2 g/(m^2 hr)

Respiratory H_2O loss = $\dot{V}_{E_{BTPS}} \left[\begin{matrix}\text{density } H_2O \\ 34C \text{ sat}\end{matrix} - \left(\begin{matrix}\text{density } H_2O \\ 21C \text{ sat}\end{matrix} \times \phi_a \right) \right] \times 60/m^2$ g/(m^2 hr)

Sweat loss = (Total H_2O loss)—(Respiratory H_2O loss)
g/(m^2 hr) —[Insensible water loss of 18g/(m^2 hr)
(Benedict and Benedict 1927)]

Mean body temp (T_b) = $0\ 8T_{re} + 0\ 2\bar{T}_s$

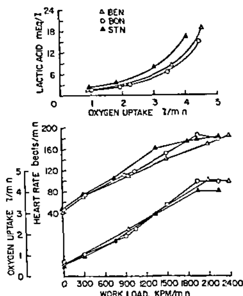
TABLE II. Comparison of circulatory and respiratory variables during continuous and intermittent work.

Subj	Work load, kpm/min	$\dot V_{O_2} \times 100$ / $\dot V_{O_2\,max}$ %	Eff %	$\dot V_{O_2}$ l/min	R_E	$\dot V_{E_{BTPS}}$ l/min	$\dot V_{E_{BTPS}}$/(f) l/br	Lactic acid mEq/l	Heart rate, 15–60"	Heart rate, 50–60"	$\dot V_{O_2}$ l/min
Continuous											
BEN	1200	60.5	24.1	2.71	0.82	51.42	2.64	1.4	135	135	0.29
BON	1200	59.2	24.3	2.63	0.87	53.71	2.16	2.5	132	134	0.27
STN	1110	62.9	23.5	2.54	0.85	59.42	1.80	3.4	161	165	0.27
Mean	1170	60.9	24.0	2.63	0.85	54.85	2.20	2.4	143	145	0.28
Intermittent											
BEN	1095	59.8	21.8	2.68	0.84	55.21	2.36	1.8	146	146	0.26
BON	1095	60.3	21.7	2.68	0.85	60.40	1.98	2.4	137	142	0.26
STN	1065	66.1	21.0	2.67	0.92	80.27	2.05	6.5	155	160	0.27
Mean	1085	62.1	21.5	2.68	0.87	65.29	2.13	3.6	146	149	0.26
% Δ	−7.3	+1.9	−10.4	+1.9	+2.3	+16.0	−3.2	+33.3	+2.0	+2.7	−7.1

Tissue conductance was calculated from the heat loss from conduction, radiation and sweating divided by the difference between the average T_{re} and T_s during the last 20 min of exercise. The net metabolic heat production was the difference between the gross metabolic heat production calculated from the O_2 uptake and the heat loss due to the external work. Evaporative heat loss was the sum of expired heat loss plus sweat heat loss.

Results

Data on the workloads and metabolic and circulatory variables are presented in Table II. For comparative purposes the intermittent workload was divided by two (i.e., 1095 kpm/min equalled 2190 kpm/30 sec plus 30 sec rest) and was 7.3 % less than for continuous work. The difference was probably due to the decreased efficiency during intermittent work caused by the additional force necessary to start the ergometer flywheel. The average efficiencies of 21.5 % for intermittent and 24.0 % for continuous work agree with those reported previously from this laboratory under similar working conditions (Åstrand *et al.* 1960 a). The average gross $\dot V_{O_2}$, rel. $\dot V_{O_2}$ and heart rates were essentially equal comparing continuous and intermittent work values. Average ± S.D. intermittent work heart rates from the last 15 sec of the rest and exercise periods were: at 3 min → 112 ± 14 and 139 ± 7; at 15 min → 131 ± 10 and 147 ± 5; *at 30 min → 138 ± 8 and 151 ± 6*; and at 50 to 60 min → 139 ± 9 and 153 ± 7 beats/min, respectively. The average lactate was elevated 1.2 meq/l in intermittent work (Table 2) and was elevated significantly above that in continuous work during the last 30 min of exercise (Fig. 2). Lactate concentration during continuous work rose initially and then decreased to resting levels at the end of work. While average $\dot V_{O_2}$ and heart rate increases were similar comparing the 2 work regimes, it appears intermittent work was more demanding upon the anaerobic metabolism.



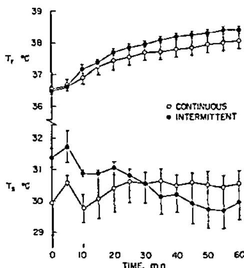


Fig 2

Fig 3

Fig 2 Average (±S.E.) oxygen uptake, heart rate and lactic acid concentration during continuous and intermittent exercise

Fig 3 Average (±S.E.) mean skin (T_s) and mean rectal (T_{re}) temperatures during continuous and intermittent exercise

There was no sharp leveling off of the two T_{re} curves and both continued to rise, but more slowly, over the last 20 min of exercise (Fig 3) The Δ T_{re} of +1.91° C during intermittent work was 0.41° C higher than in continuous work (Table III) Rectal temperature at 55 to 60 min was 0.35° C higher in intermittent work while Δ T_s of—1.56° C was markedly greater (by 2.13° C) than in continuous work (Table III) There was large inter-indivudual variability in $\overline{T}_s$ After the first 10 min of exercise the two $\overline{T}_s$ curves reacted oppositely Skin temperature during intermittent work was 1.36° C higher at 0 min than continuous (due to *subject* BEN) and, after a slight rise, dropped steadily throughout the exercise period The continuous work $\overline{T}_s$ rose initially, then dropped, rose again and stabilized at about 30.5° C (Fig 3) It is not clear why the intermittent work $\overline{T}_s$ progressively dropped because total sweat loss was somewhat less than in continuous work (Table IV) which should lead to higher Ts There were slightly lower tissue conductances (Table III) reflecting, presumably, decreased peripheral blood flow, which may have inhibited sweating

During intermittent work the average V_{O_2} was 1.9 % greater while M_{net} was 5.6 % greater than in continuous exercise (Table IV) The proportionately greater M_{net} was due to the lower mechanical efficiency, *i e* a reduced amount of work (heat) put into the bicycle would result in a larger amount of heat available for dissipation The lower sweat loss with intermittent work [32/(m² hr)] resulted in an average decreased evaporative heat loss of 18.6 kcal/ m² hr) Assuming the body specific heat

TABLE III Comparison of body temperatures and tissue conductance during cont nuous and intermittent work.

Subj	T_{r_0} C	$T_{r_{55\ 60}}$ C	ΔT_r C	T_{s_0} C	$T_{s_{55\ 60}}$ C	ΔT_s C	T_{mb_0} C	$T_{mb_{55\ 60}}$ C	ΔT_{mb} C	Tissue conductance kcal/(m² hr °C)
Continuous										
BEN	36 12	37 62	+ 1 50	27 85	29 68	+ 1 83	34 47	36 01	+1 57	42 5
BON	36 84	38 06	+ 1 22	30 68	30 87	+ 0 19	35 61	36 62	+1 01	45 3
STN	36 64	38 43	+ 1 79	31 24	30 92	— 0 32	35 56	36 93	+1 37	43 9
Mean	36 53	38 04	+ 1 50	29 92	30 49	+ 0 57	35 21	36 53	+1 32	43 9
Intermittent										
BEN	36 65	38 47	+ 1 91	31 77	28 74	— 3 03	35 60	36 52	+0 92	32 7
BON	36 40	38 36	+ 1 96	30 75	30 66	— 0 09	35 27	36 83	+1 55	47 7
STN	36 50	38 35	+ 1 85	31 63	30 08	— 1 55	35 53	36 70	+1 17	40 8
Mean	36 49	38 39	+ 1 91	31 38	29 38	— 1 56	35 47	36 68	+1 21	40 4
% Δ	—0 1	+0 9	+ 21 5	+4 7	—3 6	— 373 7	+0 7	+0 4	—8 3	—7 8

to be 0 83, the decreased heat loss of 18 6 kcal/(m² hr) would account for a body temperature increase of 0 58° C Thus, reduced sweating probably accounted for the 0 35 to 0 41° C increase in the intermittent work T_r

Discussion

Rectal temperature was selected as the measure of core temperature because it is usually more stable than either esophageal or tympanic membrane temperatures especially when work is frequently stopped and started (Robinson *et al* 1965) Also there is little difference (< 0 16° C) between equilibrium levels of T_{es} and T_{re} at rel V_{O_2} between 50 % and 70 % (Greenleaf *et al* 1969 Saltin and Hermansen 1966)

TABLE IV Water loss gross and net heat product on during cont nuous and interm ttent work

Subj	Total wt loss g hr	Sweat loss g (m² hr)	Respir H_2O loss g/(m² hr)	$+M_{gross}$ kcal/(m² hr)	$+M_{net}$ kcal/(m² hr)	J kcal/(m² hr)
Continuous						
BEN	735	318	47	406 3	308 4	—211 9
BON	710	312	52	424 4	321 3	211 3
STN	755	341	58	411 6	314 9	—231 3
Mean	733	324	52	414 1	314 9	—218 2
Intermittent						
BEN	690	288	50	401 1	316 0	196 0
BON	715	313	55	433 4	339 3	—215 [illegible]
STN	700	274	78	437 7	345 7	—201 1
Mean	702	29[illegible]	61	425 1	333 7	—20[illegible] 2
% Δ	—4.2	9 9	+ 14 8	+ 2 6	+ 5 [illegible]	— 9 4

The purpose of the present study was to investigate the temperature regulation responses during continuous and intermittent exercise independent of the effect of increased metabolic rate and heat production. Since the increased T_{re} of 0.35° C during intermittent can be explained by reductions in evaporative heat loss is is not necessary to postulate the existence of a mechanism that responds to proprioceptor and/or mechanoreceptor afferent stimuli of increased intensity that act directly in controlling the equilibrium level of core temperature. Nielson could not detect any significant elevation in T_r or esophageal temperature in relation to energy production with 30 sec work rest cycles (Nielsen 1968). However, in both Nielsen's study and the present study, the average afferent stimulation was the same during continuous and intermittent exercise because, in the latter, the load was about double but the work time was half due to the equal work rest cycles. In the present study the diminished evaporative heat loss with intermittent work could account for the increased T_{re}. A significant portion of the control of heat balance during exercise resides in the heat dissipating mechanisms, *i.e.*, sweat evaporation and conduction radiation heat losses influenced by peripheral blood flow and skin temperature. Skin temperature is the net result of ambient temperature, evaporative cooling and peripheral blood flow, but their interrelationships during exercise are obscure. The observation by I. Åstrand (1960) that the equilibrium level of T_{re} during continuous exercise was proportional to the rel. V_{O_2} rather than to the absolute V_{O_2} has been confirmed by Saltin and Hermansen (1966) who observed, in addition, that the equilibrium level of core and skin temperatures in different subjects was independent of two-fold differences in heat production. The relatively constant level of core temperature was maintained by appropriate adjustments in the rate of sweating.

In the present study it is possible the higher T_{re} with intermittent work was due, in part, to impeded heat dissipation resulting from the peripheral vasoconstriction that occurs at the beginning of relatively heavy exercise (Bishop *et al.* 1957, Christensen *et al.* 1942). If sweat rate follows T_s (Nielsen and Nielsen 1965) and T_s follows peripheral blood flow (Hertzman 1953) then one might expect decreased T_s and sweat rates with intermittent work. In the present study during intermittent work, T_s decreased progessively during the exercise period and the average total sweat rate and average tissue conductance were all less than in continuous work. However, the average tissue conductance may not be indicative of the time changes in peripheral blood flow during the intermittent work and rest period. With intermittent work of short duration the decreased skin temperatures could be explained by the action of adrenal medullary hormones particularly noradrenaline that would act to constrict the vessels in the skin. Saltin (personal communication) has observed an 8 fold increase in plasma noradrenaline concentration (from 1 $\mu g/l$ to 8 $\mu g/l$) from rest to supramaximal exercise (125 % of maximum) while Euler (1953) observed urinary noradrenaline to increase 2 times at a V_{O_2} of 2.4 l/min and 16 times the resting level when oxygen consumption rose above 4.0 l/min. In the present study the relative V_{O_2} during the intermittent work periods averaged 115 % and the average V_{O_2} was 2.7 l/min, so it is likely that plasma noradrenaline levels were also elevated. At rest, the adrenaline-

noradrenaline mixture disappears from plasma at the approximate rate of 60 % per minute (Euler 1953) If this same rate occurs during heavy exericse, the 30 sec work rest intervals would allow *ample time for a build up of the noradrenaline concentra* tion, which, in turn could cause peripheral vasoconstruction, a reduced peripheral blood flow, impeded heat transfer to the skin lowered skin tempeature and reduced sweating and evaporative heat loss Since adrenaline injected systemically, inhibits eccrine sweating it is probable that increased endogenous catecholamine concentra tions during intermittent work contributed to the reduced sweating

In the intermittent work experiments there would also be a tendency for blood to pool in the legs during the 30 sec rest periods similar to that in passive vertical tilting Indeed, Nielsen *et al* (1939) observed a rise in T_{re} and a fall in T_s subjects tilted 45° which they attrubuted to a general peripheral vasoconstriction attempting to shunt blood into the central circulation to help maintain a falling cardiac output In those experiments there was an increased sweating associated with the higher T_{re} in the tilted position, while in the present study there was a decreased sweating with the higher T_{re} in intermittent work In the latter experiments it is possible the reduced *leg motion during the rest periods contributed to the reduced cooling* but the rather strong air motion from the fans plus the nearly complete evaporation of sweat would have minimized that error Nielsen s orthostatic peripheral vasoconstriction observa tions would be difficult to substantiate during exercise due to the time delays involved between changes in peripheral blood flow and T_s combined with the augmenting ef fect of beginning exercise on sweat rate (Beaumont and Bullard 1966) The lower T_s and higher T_{re} in the present study during the last 20 min of intermittent exercise would fit with Nielsen *et al* (1939) observations

The equilibrium concentration of lactic acid of 3 8 to 4 0 meq/*l* during intermittent work was about twice as great as those observed by I Åstrand *et al* (1960 a) who uti zed 30 sec work rest cycles at relative oxygen uptakes of 95 to 100 % of maximum In the present study the average work intensity was 115 % of maximum and the higher lactates were due in part to the greater loads During maximal and supra maximal intermittent exercise the blood lactate concentration is proportional to the *length of the work periods with 3 min work rest cycles the lactates reach 13 2 meq l* I Åstrand *et al* 1960 a At continuous maximal work tissue lactate concentration is 20 to 30 % greater than blood lactate concentration and following exercise of 3 to 4 min duration blood lactates rise for 5 to 10 min due to diffusion from the tissue cells into the blood Åstrand and Rodahl 1970 During intermittent exercise with equal work rest cycles of 30 sec and less there is no large accumulation of blood lactate at maximal and supramaximal workloads Therefore during these short work rest cycles there is either (a) a reduced buildup of lactate during the work period with more or less constant removal during the rest period (b) a constant high level pro duction during the work period but accelerated removal during rest or c a com bination of (a and b above I Åstrand *et al* 1960 b and Christensen *et al* 1960 have settled on hypothesis (a) above and have suggested the work was performed essentially aerobically with the oxygen supply in the muscle bound to myoglobin

and to hemoglobin and the oxygen coming into the muscle with the incoming blood being able to oxidize the substrates sufficiently to keep the lactate concentration low The low average ventilatory exchange ratios (Table II) would also indicate aerobic metabolism Myoglobin has been suggested as the contributor of an extra oxygen store that allows the work to be performed aerobically (I Åstrand et al 1960) The classical interpretation of the function of myoglobin is to act as a ' catalyst" to facilitate transfer of oxygen from hemoglobin to the cellular enzymes (Prosser 1961) Since myoglobin has a much greater affinity for O_2 than hemoglobin (dog myoglobin maintains 60 % oxygenation at 5 mm Hg pressure of O_2, while blood hemoglobin maintains only 5 % saturation at the same pressure—Prosser 1961), it is difficult to envision myoglobin as the most significant oxygen store to be drawn upon at the beginning of heavy exercise From calculations of the estimaged O_2 bound to myoglobin, I Åstrand et al (1960 b) could account for slightly less than half the O_2 supply needed to perform aerobically 10 sec work rest cycles at 2,520 kpm/min

Regarding hypothesis (b) above, as the intermittent work progresses, there would be an increased muscle and systemic blood flow to the liver where lactate oxidation would be facilitated In the present study during intermittent and continuous work, the lactate concentrations increased about 2 fold, indicating anaerobic metabolism present It is likely myoglobin plays an important role for O_2 transfer during the first few seconds of muscular exercise but, as the work period lengthens the transfer function of myoglobin reaches an upper limit and then the equilibrium concentration of blood lactate reflects the balance between the aerobic and anaerobic processes

Since the total rate of heat production in the present study was essentially the same in the continuous and intermittent experiments but the latter T_{re} was higher, it is possible that during intermittent work the level at which body temperature is regulated is related non linearly to total work rate Exercise without thermoregulatory adjustments would cause large increases in body temperature Regulatory adjustments result from changes in body temperature, and possibly other factors Intermittent exercise permitted a higher increase in body temperature and was associated with a reduced sweat rate This would indicate a reduced efficiency of the thermoregulatory system Reflex circulatory adjustments at the onset of exercise, or non linearity in the contribution of non thermal inputs at work rates above 100 % of max V_{O_2} could account for the relative inefficiency Further research is needed to determine if this hypothetical non linearity could be due to a function of the duration and/or intensity of the work and rest cycles, hormonal changes, or to some other factor not specifically related to the working situation, such as posture

noradrenaline mixture disappears from plasma at the approximate rate of 60 % per minute (Euler 1953) If this same rate occurs during heavy exercise, the 30 sec work rest intervals would allow ample time for a build up of the noradrenaline concentration, which, in turn, could cause peripheral vasoconstriction, a reduced peripheral blood flow, impeded heat transfer to the skin, lowered skin tempeature and reduced sweating and evaporative heat loss Since adrenaline, injected systemically, inhibits eccrine sweating, *it is probable that increased endogenous catecholamine concentrations during intermittent work contributed to the reduced sweating*

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From the Department of Pharmacology, School of Medicine, Linköping, Sweden

Relaxing and Metabolic Actions of ACTH in Rabbit Colon

By

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Received 31 March 1970

Abstract

Andersson, R., E. Mohme-Lundholm, N. Svedmyr and N. Vamos *Relaxing and metabolic actions of ACTH in rabbit colon* Acta physiol scand 1971 *81* 11—17

ACTH in the dose range of 0 15—0 60 IU/ml relaxed the circular muscular layer of rabbit colon contracted by carbacholine The content of cyclic AMP was doubled There was an activation of phosphorylase *a* which gave the same dose effect and time-effect curves as the relaxing [illegible] creased [illegible] β-recepto[illegible] of ACT[illegible] hydrolys[illegible] of isopre[illegible] prenaline, ACTH did not decrease the adenosine triphosphate content of the muscle It is suggested that the relaxing action of ACTH is mediated by the adenyl cyclase cyclic AMP system

The stimulation of adenyl cyclase, leading to the formation of cyclic AMP has been shown by Sutherland and coworkers (*cf* Sutherland Butcher and Robison 1968) to mediate metabolic and pharmacological actions of catecholamines and some peptide hormones such as ACTH, TSH vasopressin and glucagon In some tissues the receptors of adenyl cyclase have a high specificity and are only stimulated by one agonist In adrenal cortex only ACTH increases the production of cyclic AMP In other tissues the specificity of the adenyl cyclase receptors is less prominent and many different agonists are effective In adipose tissue the catecholamines, serotonine, ACTH, TSH, vasopressin and glucagon have a lipolytic action probably as a consequence of an increased production of cyclic AMP

In studies on rabbit colon we found that a relaxing action of catecholamines was produced by stimulation of both adrenergic α- and β-receptors but that different mechanisms were involved (Andersson and Mohme-Lundholm 1969) The relaxation produced by isoprenaline through selective stimulation of β receptors was combined with an increase in cyclic AMP, an activation of phosphorylase *a*, an increase in the carbohydrate metabolism and a reduction of the high energy phosphate com-

ponds of the muscle The relaxing and metabolic actions of isoprenaline could be completely reproduced by addition of cyclic AMP Theophylline and puromycin which inhibit the enzymatic hydrolysis of cyclic AMP, potentiated the relaxing action of isoprenaline, whereas an adrenergic β-receptor blocking agent, sotalol, which inhibits the formation of cyclic AMP, blocked the relaxing action of isoprenaline It was suggested that the relaxing action produced by adrenergic β-receptor stimulation was mediated by cyclic AMP and dependent partly on phosphorylase *a* activation and partly on an ATP ase activating effect (Andersson and Mohme-Lundholm 1970)

In order to obtain further evidence for this hypothesis and more information on the role of cyclic AMP in smooth muscle relaxation, we studied in rabbit colon the effect of some of the peptide hormones (ACTH, vasopressin, glucagon, TSH) which in other tissues stimulate adenyl cyclase Of these hormones ACTH glucagon and vasopressin had a relaxing action on the rabbit colon The relaxing action of glucagon *and vasopressin was probably not mediated by adrenergic β receptor stimulation* alone The mechanisms of these relaxing actions are discussed in another paper (Andersson 1969) ACTH however, produced a relaxing effect via stimulation of an adrenergic β-receptor mechanism The relaxing and metabolic effects of ACTH were therefore studied more completely and compared with those of isoprenaline and cyclic AMP

Methods

The tests were made on isolated rabbit colon suspended in 30 ml Krebs Henseleit bicarbonate buffer solution at 37° C and bubbled with 95 % O_2 + 5 % CO_2 The mucosa layer was carefully removed *The changes of the tension of the circular muscle layer were recorded with an* [illegible] 03 tension transducer on a Grass polygraph The muscle was first suspended for 60 min in buffer solution with a normal glucose content (11 5 × 10^{-3} mole/l) in order to obtain constant values of phosphorylase *a* and high energy phosphate compounds The preparation was successively lengthened until a constant tension of 1 p was obtained The Krebs Henseleit solution was then changed to a glucose free solution and 2 5 × 10^{-7} g/ml of carbacholine was added to increase the tension to about 10 p After 10 min ACTH L-isoprenaline or cyclic AMP was added When the effect of ACTH was blocked by an adrenergic β receptor blocking agent sotalol the drug was added 5 min before ACTH

The muscle preparation was then frozen in Frigen 11 at −80° C On one third of the muscle the phosphorylase activity *a* without AMP and the total activity with 0 001 M AMP was determined according to Barding *et al* 1962 The rest of the muscle was homogenized in 6 % ice-cold perchloric acid centrifuged and neutralized with K_2CO_3 and centrifuged once more In the extract adenosine triphosphate ATP creatine phosphate (CrP) glucose 1 phosphate G 1 P glucose 6-phosphate G 6-P fructose 6-phosphate (F-6-P) and fructose 1 6-phosphate F 1 6-P were determined by enzymatic methods as described in another paper (Andersson and Mohme Lundholm 1970 The ACTH preparation used was Isactid® (Ferring which is a crystalline chromatographic purified preparation from hog hypophysis In separate muscle preparations the effect on the cyclic AMP content was determined according to Kakiuchi and Rall 1963

Results

Relaxing action of ACTH ACTH in a concentration of 0 15 0 60 IU/ml produced dose dependent relaxation which started after a latency period of about 20 sec and reached a maximum after about 3 min *Even with the highest dose of ACTH the*

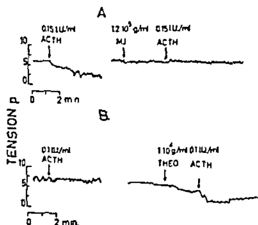


Fig 1



Fig 2

Fig 1 The muscle layer from rall t colon . . . ate l ff and bubbled with 95 . . . e circula muscle layer The ten . . . ll e effe of sotalol and theoph . . . f ACTH in a concentrat on of (. . . B ACTH in a concentration of . . . ldition of theophylline (1×10⁻⁴ g/ml) ACTH in the same c ncentration induced a markedl , tentiated relaxant effect

Fig 2 Dose response curve of ACTH Influence of sotalol Concentration of ACTH in log scale Maximal relaxation = 100 per cent

tension decrease was only about 30—40 per cent of the initial tension i e from about 10 p to 7—6 p The duration of the relaxation was at least 15 min after which the tension started to rise slowly

The relaxing action of ACTH in a concentration of 0 15 IU/ml was completely blocked by an adrenergic β receptor blocking agent (sotalol) in a concentration of $1\ 2\times10^{-3}$ g/ml i e the same concentration that blocked the action of isoprenaline (Fig 1) In a higher concentration ACTH completely overcame the blocking action of sotalol, the type of inhibition was competitive (Fig 2)

The relaxing action of ACTH was markedly potentiated by theophylline (1×10^{-4} g/ml) and puromycin ($7\ 5\times10^{-5}$ g/ml) (Fig 1) which drugs also potentiate the action of isoprenaline (Andersson and Mohme Lundholm 1969)

The action of ACTH was weakened but still present in a medium where the Na in Krebs Henseleit solution had been replaced by K (145 meq/l) When the buffering capacity of the muscle was increased by adding $NaHCO_3$ to the Krebs Henseleit buffer solution the relaxing action of ACTH was blocked

Metabolic actions of ACTH in colonic muscle ACTH (0 3 IU/ml) had increased the cyclic AMP content of the muscle from a basal value of $2\ 5\pm0\ 23\times10^{-10}$ mole/g wet weight by $2\ 0\pm0\ 46\times10^{-1}$ mole/g (n = 6 $P<0\ 01$) 90 sec after its addition

ACTH significantly increased the phosphorylase *a* activity 60 sec after its addi

TABLE I Influence of different doses of ACTH on relaxation (decrease of tension in per cent of

Drugs	n	Relax per cent	Phosphorylase *a* per cent	G-1-P	G 6 P	F 6 P	F-1-6 P	ATP	CrP
Control values	6		5 2 ± 1 4	16 ± 2	41 ± 5	13 ± 1	29 ± 4	115 ± 25	38 ± 7
ΔACTH 0 15 IU/ml	6	17 ± 1***	1 9 ± 06*	4 ± 1**	7 ± 2**	2 ± 1	7 ± 1***	22 ± 21	11 ± 6
ΔACTH 0 30 IU/ml	6	22 ± 3***	2 0 ± 05**	3 ± 1*	10 ± 5	3 ± 1*	7 ± 3*	12 ± 20	17 ± 3**
ΔACTH 0 60 IU/ml	6	31 ± 5***	6 0 ± 2 5*	4 ± 1**	8 ± 6	3 ± 1*	13 ± 3**	28 ± 22	11 ± 4*
Control values	6		3 8 ± 0 8	22 ± 3	34 ± 6	15 ± 2	33 ± 14	95 ± 14	31 ± 6
Δ1 ACTH 0 3 IU/ml	6	23 ± 2***	2 0 ± 0 8*	2 ± 1	8 ± 6	3 ± 2	5 + 2*	1 ± 8	11 ± 2**
Sotalol control	6	12 ± 3	3 8 ± 0 5	22 ± 4	45 ± 9	16 ± 1	28 ± 8	87 ± 14	36 ± 5
Δ2 (Sotalol + ACTH Sotalol)	6		−0 4 ± 0 4	−1 ± 1	−5 ± 4	1 ± 1	−2 ± 2	−1 ± 11	−3 ± 6
Δ (1 3)		23 + 2***	2 4 ± 0 9*	3 ± 1**	13 ± 8	2 ± 2	7 ± 2**	2 ± 14	14 ± 6*

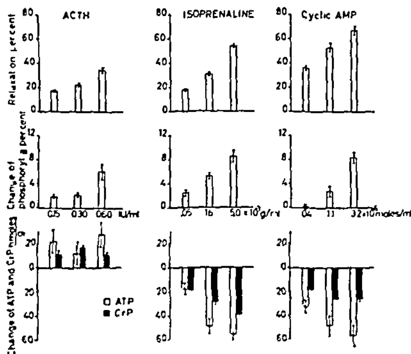


Fig 3 Dose response curves for the effect of ACTH *L*-isoprenaline (free base) and cyclic AMP on relaxation phosphorylase *a* activity and the content of high-energy phosphate compounds in colonic muscle The determinations were performed 60 sec after addition of isoprenaline and cyclic AMP and 3 min after addition of ACTH Mean ± S E M ACTH tests n = 6 Isoprenaline tests n 7 Cyclic AMP tests n 5

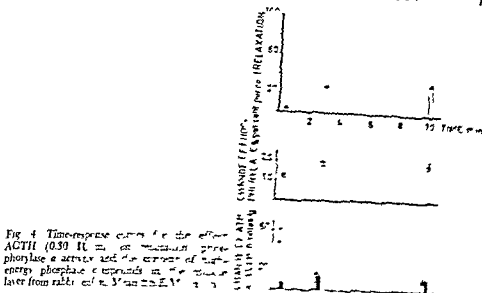


Fig. 4. Time-response curves for the effects of ACTH (0.30 IU ml) on maximum [illegible] phosphorylase *a* activity and the contents of high-energy phosphate compounds in the muscle layer from rabbit colon. Mean ± S.E. [illegible]

tion. There was a dose-dependent [illegible] phosphorylase *a* increase [illegible] (Table I) [illegible] changed. There was a significant [illegible]

The relaxing and metabolic actions of [illegible] and cyclic AMP. Fig. 3 shows a [illegible] In contrast to ACTH, noradrenaline [illegible] ATP and CrP [illegible] noradrenaline after a [illegible] high energy phosphate compounds [illegible] the metabolic actions of ACTH. At [illegible] reduce the ATP or CrP content (Fig. 4) [illegible]

After pretreatment of the muscle with [illegible] actions of ACTH were completely blocked. [illegible] tween the effect of ACTH with and without [illegible] (Table I) [illegible]

There was a significant correlation between the relax[illegible] a dose of 0.60 IU ml and its phosphorylase *a* act[illegible] regression was Y = 2.46 X − 13.1, r = 0.9, P<0.001 [illegible]

Discussion

ACTH relaxed rabbit colon and increased the cyclic [illegible] phosphorylase *a* activity and there was a significa[illegible] and phosphorylase activating actions. The [illegible] relaxing and metabolic actions were competit[illegible] tor blocking agent and the former action was [illegible]

mycin In these respects the action of ACTH showed striking similarities both quantitatively and qualitatively with those of isoprenaline It seems probable that ACTH and isoprenaline stimulated the same or two very similar receptors in smooth muscle

There was one difference between the effects of ACTH and isoprenaline Isoprenaline reduced the contents of ATP and CrP initially but after a latency period of 10 min increased these contents ACTH had no reducing action on the high energy phosphate compounds From the beginning it increased the CrP content of the muscle

It is probable that the metabolic and relaxing actions of isoprenaline were mediated by cyclic AMP In the lowest active concentration (0.36×10^{-6} mole/ml) cyclic AMP reduced the ATP content Only in a higher concentration (1.1×10^{-6} mole/ml) did cyclic AMP increase the phosphorylase *a* activity indicating that cyclic AMP had two independent metabolic effects It is rather probable that the relaxing and metabolic actions of ACTH were also mediated by cyclic AMP, but that the cyclic AMP produced via ACTH stimulation had only a phosphorylase *a* activating effect Cyclic AMP in the lowest active concentration reduced the ATP content It is therefore improbable that the reason for the difference between the metabolic actions of ACTH and isoprenaline was that ACTH had a weaker stimulating action on adenyl cyclase than isoprenaline We consider it more probable that there are two kinds of adenyl cyclases present in the smooth muscle cell These enzymes may either have a different location or different receptors One of these may mediate the phosphorylase *a* activation of ACTH and isoprenaline the other the ATPase activating effect of isoprenaline

According to Davoren and Sutherland (1963) adenyl cyclase is located in the cell membrane In rabbit skeletal muscle Rabinowitz *et al* (1965) demonstrated adenyl cyclase activity in the microsomal fraction also and this activity has recently been found in the mitochondria of platelets (Marquis Vigdahl and Tavormina 1969) If adenyl cyclase is located in different parts of the smooth muscle cell it is likely that ACTH with a large molecule is only able to reach the adenyl cyclase located in the cell membrane whereas isoprenaline with a smaller molecule could reach an intracellularly located enzyme Further studies are needed to clarify this question

ACTH also relaxed bronchial smooth muscle from the rabbit In isolated human bronchial muscle it had a relaxing action which was markedly potentiated by theophylline This observation is of pharmaco-therapeutical interest as ACTH in combination with theophylline is used as bronchodilative therapy in more severe cases of bronchial asthma Svedmyr *et al* 1970

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Conjoint Action of Sodium and Angiotensin on Brain Mechanisms Controlling Water and Salt Balances

By

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Abstract

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The effects on water and salt balances of a simultaneous infusion of angiotensin and hypertonic NaCl into the 3rd brain ventricle were studied in goats in normal water balance and in hydrated animals. For comparison similar infusions of angiotensin alone (solved in slightly hypotonic saline) and of hypertonic NaCl were made. Like the hypertonic NaCl, 30 min infusions of angiotensin alone induced drinking in animals in normal water balance, and an inhibition of the water diuresis in the hydrated goat. The simultaneous infusion of both substances resulted in a marked potentiation of the dipsogenic and the antidiuretic effects. A possible explanation may be that angiotensin facilitates the transport of Na^+ into brain cells regulating thirst and ADH release, and that the intracellular Na^+ concentration rather than strictly osmotic factors determines the activity of these cells. In the hydrated goat a central effect of angiotensin strongly enhanced the natriuretic response to intraventricular infusions of hypertonic NaCl, and extreme natriuresis developed as result of the combined infusions in hydrated, salt-supplemented animals. During normal water balance this sodium-angiotensin synergism was less obvious, which suggests that both an expanded fluid volume and an elevated intracellular Na^+ concentration are needed for optimal activation of a brain mechanism which stimulates renal Na^+ excretion. The time course of the centrally induced natriuresis indicates that the effect on the kidney may be mediated by a humoral agent.

A preliminary report has been given of experiments in the goat which demonstrate a synergistic action of angiotensin and hypertonic NaCl on brain mechanisms controlling water and salt balance (Andersson and Westbye 1970). The study was performed in animals maintained on extra dietary salt. These experiments are described in detail here together with further experiments which show that the same synergism is also seen in [illegible] supplemented animals.

Methods

Animals and animal care. [illegible] female goats (b.w. 30 to 40 kg) were used for repeated experiments over a period of [illegible] months. The animals were routinely confined in metabolism cages and

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all experiments were conducted in these cages The goats had free access to chaffed hay and water at a temperature of 20±1° C In addition they were given 400 g/day of commercial grain mix Four of the goats received 6 g of NaCl every afternoon The salt was either consumed together with the grain mix or was drunk as warm 6 % NaCl solution The morning refill of the hay bins was made at 8 a m The intraventricular infusions were not started until 2 hrs later, i e at a time when the goats had eaten to satisfaction and had completed the postprandial drinking

Brain implantations and infusion technique The goats were prepared with permanent cannulae in the anterior part of the 3rd brain ventricle To avoid the disadvantage of having a dead space , the infusions were performed via an inner cannula The inner cannula was filled with the solution to be infused and was inserted to the ventricular end of the permanent cannula before the infusion was started The operation and infusion techniques have been described in detail earlier (Andersson Olsson and Warner 1967) In all experiments cerebrospinal fluid was observed to drain out of the permanent cannula on compression of the neck of the animal before insertion of and after removal of the inner cannula Thus a completely free mixing of the infused solution with the fluid of the ventricle was guaranteed

s always maintained at 10 μl/min The follow
l ventricle
in slightly hypotonic (0 12 M) saline (in the
)
0 5 M) NaCl solution

were started

Urine collection and analyses The urine was collected in 10 or 20 min samples via a retention catheter inserted into the urinary bladder Urine Na and K⁺ were determined by use of an EEL' flame photometer A 'Knauer osmometer was used for determinations of urine osmolality

Planning of the experiments

In each animal the experiments were grouped in series of three 30 infusions performed with a minimum interval of 3 days The infusions in a series consisted of angiotensin alone of
solution The mutual
infusions the dose of

usions were made in
5 of the goats when not pre hydrated 3 of the animals were salt supplemented The amounts
series) 1 ng (3 series) and 0 5 ng
series
infusions and one incomplete series
ng hydration All 6 goats were used
d per kg b w and min were ? ng
(2 series) and 1 3 ng (7 series) The hypertonic NaCl used was 0 5 M (4 series) and 0 33 M (5 series)

Exceptions from this scheme were made on 2 occasions in 2 of the salt supplemented goats during hydration Repeated brief (5 min) infusions of all 3 kinds of solutions were then made to test for antidiuretic effect

Results

A Thirst

No difference was observed between the dipsogenic effect of the intraventricular infusions in the salt supplemented animals and in the goats not receiving extra dietary salt Hydration on the other hand changed the water consumption and the drinking pattern of the animals characteristically

Normal water balance In all series of experiments in not pre hydrated goats the total amount of water drunk during the combined infusion of angiotensin and hyper-

tonic NaCl was greater than the sum of the water consumption during the infusions of angiotensin alone and of the 0.33 M NaCl solution. The stronger dipsogenic effect of the combined infusions was also evidenced by a shorter latency time before the onset of drinking (average 2 min as compared to 6 min for angiotensin alone and 7 min for 0.33 M NaCl). The average amounts of water drunk (mean ± standard error of mean) during the different infusions in the not pre-hydrated animals were the following:

Infusion	*Water intake* ml/kg b.w.	*Number of expts*
Angiotensin alone	47 ± 5	9
Angiotensin in 0.33 M NaCl	123 ± 9	9
0.33 M NaCl	15 ± 3	9

Hydration. The combined intraventricular infusions of angiotensin and hypertonic NaCl acted strongly dipsogenic also in the pre-hydrated goat. The latency time for drinking was about twice that observed when the same animal was in normal water balance. The goats drank eagerly and to prevent water intoxication the water had to be withheld when the consumption had exceeded 50 ml/kg b.w. In 4 series of experiments no drinking occurred in the pre-hydrated animals during the infusions of angiotensin alone or of merely the hypertonic NaCl solution. The animal drank during these infusions in the other 5 series, but the drinking took place during the second half of the infusion period and relatively small amounts of water were consumed. The average drinking during the different intraventricular infusions in the pre-hydrated goats was the following:

Infusion	*Water intake* ml/kg b.w.	*Number of expts*
Angiotensin alone	10 ± 7	9
Angiotensin in hypertonic NaCl	>50	9
Hypertonic NaCl	7 ± 3	8

Fig. 1 shows the drinking of one of the salt supplemented goats during 2 series of experiments performed with the animal in normal water balance (*left*) and 2 series made during hydration (*right*).

B. Antidiuresis

The course of the water diuresis and the changes in urine osmolality were followed in all 9 series of 30 min infusions performed during hydration. Both in the goats maintained on extra dietary salt and in the not supplemented animals the combined intraventricular infusions of angiotensin and hypertonic NaCl inhibited the water diuresis much more effectively than did similar infusions of angiotensin alone or of

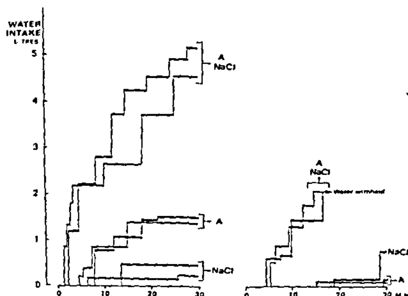


Fig 1 The drinking response of a goat to 30 min infusions into the 3rd brain ventricle of angiotensin together with hypertonic NaCl solution $\left(\frac{A}{NaCl}\right)$ and to similar infusions of angiotensin alone (A) or of merely the hypertonic saline (NaCl)

Left The dipsogenic effects of 2 series of infusions made when the goat was in normal water balance at the start of the infusions Dose of angiotensin infused = 2 ng/kg min NaCl solution = 0 33 M

Right The drinking response during hydration (100 ml of water/kg b w given 90 min before start of the infusions) Dose of angiotensin = 1 3 ng/kg min Hypertonic NaCl solution = 0 5 M

Rate of infusion in all expts = 10 μl/min Minimum interval between the infusions = 3 days

merely hypertonic NaCl solution Urine osmolality started to rise in the first 10 min collection period after the onset of the combined infusion and exceeded plasma osmolality (>280 mosm/kg) in the next 10 min sample of urine Due to a simultaneous stimulation of renal salt excretion however maximal reduction in the urine flow did usually not appear until 30 min to 1 hr after the end of the infusion period At this time urine osmolality reached its maximum (500—700 mosm/kg) The water diuresis did not reappear until about 2 hrs after cessation of the combined infusions

The 30 min intraventricular infusions of angiotensin alone did not cause obvious inhibition of the water diuresis until 20 to 30 min after the start of the infusion This inhibition did not last for more than 20 to 40 min The antidiuretic effect of the infusions of merely hypertonic NaCl solution was somewhat more pronounced than that obtained by the corresponding infusions of angiotensin alone It was however always of less magnitude and shorter duration than that induced by the combined infusions The inhibitory effects on the water diuresis of a series of 30 min infusions in one of the goats are shown in Fig 2 (expressed as changes in renal clearance of osmotically free water (C_{H_2O}))

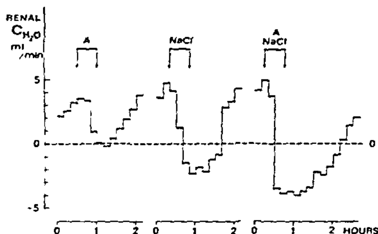


Fig. 2. Inhibition of the water diuresis in a hydrated goat induced by 30 min infusions into the 3rd brain ventricle of angiotensin alone (A), of 0.5 M NaCl solution (NaCl), and of angiotensin solved in 0.5 M NaCl ($\frac{A}{NaCl}$).

C_{H_2O} = Renal clearance of "osmotically free water". That renal clearance of free water turned negative indicates a release of antidiuretic hormone which apparently was greatest during the combined infusion.

Dose of angiotensin = 1.3 ng/kg min. Rate of infusion 10 µl/min. Three days interval between experiments.

Brief (5 min) infusions into the 3rd ventricle were employed to obtain a more distinct comparison between the antidiuretic effects of the 3 kinds of solutions. Infusions of 0.33 M NaCl, of angiotensin alone (2 ng/kg min) and of angiotensin together with 0.33 M NaCl were made alternatively in 2 of the salt-supplemented

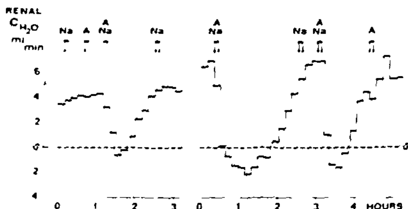


Fig. 3. Renal clearance of osmotically free water (C_{H_2O}) in two hydrated goats subjected to brief (5 min) infusions into the 3rd brain ventricle of 0.33 M NaCl (Na), of angiotensin alone (A) and of angiotensin together with 0.33 M NaCl ($\frac{A}{Na}$). Only the combined infusions reverted the free water clearance to negativity, which indicates a release of significant amounts of antidiuretic hormone (ADH). ADH was apparently not released by the brief infusions of angiotensin alone or of merely 0.33 M NaCl solution.

Dose of angiotensin = 1.3 ng/kg min. Rate of infusion 10 µl/min.

goats during hydration. As shown in Fig. 3, neither angiotensin alone nor 0.33 M NaCl inhibited the water diuresis. In contrast, the combined infusions of angiotensin and hypertonic saline elicited a temporary inhibition of the diuresis and a rise in urine osmolality above plasma level (negative C_{H_2O}). In spite of the short duration the combined infusions also evoked obvious thirst in the hydrated animals.

C. Natriuresis

The 3 kinds of 30 min intraventricular infusions all produced an increase in renal Na^+ excretion which generally reached its maximum during the 2nd post-infusion 10 min period of urine collection. A concomitant but less pronounced rise in renal K^+ excretion was also seen. The preinfusion Na^+ excretion was very low in the animals which did not receive dietary salt supplementation and the greatest relative increases in the Na^+ excretion were seen in these goats. However, conspicuous natriuresis was obtained only in the salt supplemented animals and then as effect of the combined infusions of angiotensin and hypertonic saline. This synergistic action of angiotensin and hypertonic NaCl was most evident during hydration.

Normal water balance. In most series of experiments performed on goats in normal water balance the natriuretic effect of the combined infusion was approximately equal to the sum of the effects of the infusions of angiotensin alone and of 0.33 M NaCl. However, in two series the infusions of angiotensin alone caused practically the same increase in renal Na^+ excretion as did the combined infusions. The average natriuretic effects (M ± S.E.M.) of the 3 solutions during normal water balance were the following:

Infusion	*Basal excretion* Na^+ μEq/min	*Maximal excretion* Na^+ μEq/min	*Number of expt*
Angiotensin alone	130 ± 50	440 ± 120	9
Angiot. + 0.33 M NaCl	50 ± 20	740 ± 110	9
0.33 M NaCl	50 ± 20	170 ± 40	9

Hydration. The intraventricular infusions of merely hypertonic NaCl induced a somewhat stronger natriuretic response in the prehydrated goat than in the not hydrated animal. The reverse was true for the infusions of angiotensin alone. Nevertheless, the adding of angiotensin to the hypertonic NaCl markedly potentiated its natriuretic effect in the prehydrated goats. Extreme natriuresis developed during the combined infusions in the salt supplemented animals and renal Na^+ excretion remained significantly above preinfusion level for up to 3 hrs after cessation of the intraventricular infusion. The natriuretic response to the combined infusion had then outlasted the antidiuretic effect by 1 hr. The following table summarizes the effects on renal Na^+ excretion in the hydrated salt supplemented goats:

Infusion	*Basal excretion* Na^+ μEq/min	*Maximal excretion* Na^+ μEq/min	*Number of expts*
Angiotensin alone	70 ± 40	240 ± 60	5
Angiot. + Hypertonic NaCl	110 ± 30	1160 ± 130	5
Hypertonic NaCl	100 ± 50	450 ± 160	4

The effects on renal Na^+ excretion of one series of infusions in a hydrated salt-supplemented goat are shown in Fig. 4 (*left*).

The natriuretic effect of the combined infusions was less conspicuous in hydrated goats which had not received extra dietary salt. However, also in these animals the infusions of angiotensin together with hypertonic NaCl stimulated renal Na^+ excretion much more effectively than the infusions of angiotensin alone or the infusions of merely the hypertonic saline (see table below).

Infusions	*Basal excretion* Na^+ μEq/min	*Maximal excretion* Na^+ μEq/min	Number of expts
Angiotensin alone	7 ± 1	50 ± 10	4
Angiot. + Hypertonic NaCl	7 ± 1	400 ± 100	4
Hypertonic NaCl	11 ± 5	50 ± 20	4

The effects on renal Na^+ excretion obtained in one series of infusions performed during hydration in a not salt supplemented goat are shown to the right in Fig. 4.

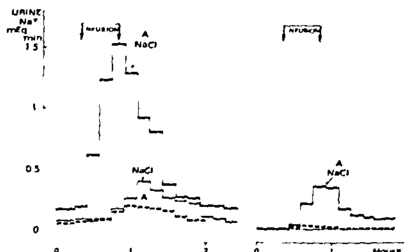


Fig. 4. Effects on renal sodium excretion in two hydrated goats of 30 min infusions into the 3rd brain ventricle of angiotensin together with 0.5 M NaCl $\left(\frac{A}{NaCl}\right)$, of angiotensin alone (A) and of merely 0.5 M NaCl solution (NaCl).

Left. A series of infusions performed in a goat receiving dietary salt supplementation (— g NaCl/day).

Right. The corresponding series of infusions made in an animal not receiving extra dietary salt.

Dose of angiotensin: 2 ng/kg min. Rate of infusion 10 μl/min. Minimum interval between infusions = 3 days.

D Intravenous control infusions

In each of the salt supplemented animals 30 min i v infusions of angiotensin (5 ng/kg min) were made when the goats were in normal water balance No drinking took place during these infusions In 2 of the goats however the i v infusions resulted in 3 and 5 times increase in renal Na excretion No significant increase was seen in the other 2 animals

In one of the salt supplemented goats and in 2 animals which did not receive extra dietary salt i v infusions of angiotensin (3 ng/kg min) were made during hydration No drinking or inhibition of the water diuresis took place In the salt supplemented animal the renal Na excretion rose from 30 to 130 μEq/min during the i v infusion No significant change in the Na excretion was seen in the other 2 hydrated goats

Discussion

The homeostatic control of body water content is dependent on a regulated release of antidiuretic hormone (ADH) from the neurohypophysis and on an efficient thirst mechanism which ensures that the water intake keeps pace with the water loss

Verney s (1947) studies form the basis for the conception of an osmometric regulation of the ADH release Verney found that a rise in the tonicity of the blood which flows through the hypothalamus may cause a release of ADH in hydrated dogs This happened when hypertonic solutions of sodium salts or of saccharose were used to increase the blood tonicity However when hypertonic urea solution was employed for the same purpose no ADH release was obtained The lack of response to urea was explained as due to the high diffusibility of the substance Verney suggested that hypothalamic osmoreceptors regulate the release of ADH and that these receptors are not stimulated by a rise in body fluid tonicity as such but rather by any change in the extracellular fluid which reduces the volume of the receptors This would explain why urea which rapidly diffuses into the cells was not found to stimulate the receptors Injections of small amounts of hypertonic NaCl solution into the anterior hypothalamus or into the 3rd ventricle were later found to elicit excessive drinking in the goat and it was suggested that a hypothalamic osmoreceptor mechanism also may participate in the regulation of water intake (Andersson 1953) The picture of the function of hypothalamic osmoreceptors appears to need revision in the light of the more recent finding that urea passes the blood brain barrier very slowly and that intravascular injections of hypertonic urea cause dehydration of the central nervous system (*cf* Kleeman and Cutler 1963) That infusions of hypertonic saccharose into the 3rd ventricle of goats do not cause ADH release or apparent thirst (Olsson 1969) also seems incompatible with the current osmoreceptor theory As distinguished from infusions of hypertonic NaCl infusions of hypertonic NH_4Cl into the 3rd ventricle are not dipsogenic in the goat (Andersson *et al* 1967) It indicates that the central thirst neurons are stimulated preferentially by the elevated Na concentration and not by the rise in the Cl concentration

Other organs than the brain are also involved in the regulation of water intake

Infusion	*Basal excretion* Na⁺ μEq/min	*Maximal excretion* Na⁺ μEq/min	*Number of expts*
Angiotensin alone	70 ± 40	240 ± 60	5
Angiot + Hypertonic NaCl	110 ± 30	1160 ± 130	5
Hypertonic NaCl	100 ± 50	450 ± 160	4

The effects on renal Na^+ excretion of one series of infusions in a hydrated, salt-supplemented goat are shown in Fig 4 (*left*)

The natriuretic effect of the combined infusions was less conspicuous in hydrated goats which had not received extra dietary salt However, also in these animals the infusions of angiotensin together with hypertonic NaCl stimulated renal Na^+ excretion much more effectively than the infusions of angiotensin alone or the infusions of merely the hypertonic saline (see table below)

Infusions	*Basal excretion* Na⁺ μEq/min	*Maximal excretion* Na⁺ μEq/min	Number of expts
Angiotensin alone	7 ± 1	50 ± 10	4
Angiot + Hypertonic NaCl	7 ± 1	400 ± 100	4
Hypertonic NaCl	11 ± 5	50 ± 20	4

The effects on renal Na^+ excretion obtained in one series of infusions performed during hydration in a not salt-supplemented goat are shown to the right in Fig 4

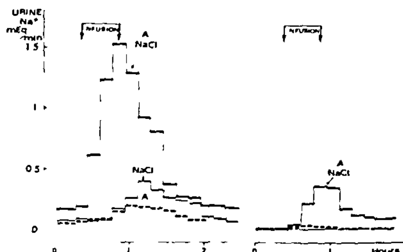


Fig 4 Effects on renal sodium excretion in two hydrated goats of 30 min infusions into the 3rd brain ventricle of angiotensin together with 0 5 M NaCl $\left(\frac{A}{NaCl}\right)$ of angiotensin alone (A), and of merely 0 5 M NaCl solution (NaCl)

Left A series of infusions performed in a goat receiving dietary salt supplementation (6 g NaCl/day)

Right The corresponding series of infusions made in an animal not receiving extra dietary salt

Dose of angiotensin 2 ng/kg min Rate of infusion 10 μl min Minimum interval between infusions = 3 days

pear to be irrelevant since the addition of minute amounts of angiotensin to the hypertonic saline does not increase its tonicity significantly A more plausible explanation would be that angiotensin mimics and strengthens the effects of an infusion of hypertonic saline into the 3rd ventricle by facilitating the transport of Na from the extracellular fluid into brain cells which participate in the regulation of thirst and the release of ADH It has been shown that angiotensin stimulates the uptake of Na^+ in frog skin (McAfee and Locke 1967) and in the mucosal cells of rat jejunum (Crocker and Munday 1970) If the same holds true for the above mentioned brain cells it may be the intracellular Na concentration (rather than strictly osmotic factors) that determines the activity of these neurons

Prolonged i v infusions of roughly twice the amount of angiotensin did not induce drinking or visible release of ADH It shows that the drinking and antidiuretic responses obtained during the intraventricular application of angiotensin reflected a central effect of the substance Nothing appears to be known about the transport of angiotensin across the blood brain barrier in the goat However even if an effective transport did take place during the i v infusions the resulting brain concentration was apparently too low for the substance in itself to activate the thirst and ADH releasing mechanisms It would be of particular interest however, to study in future experiments whether the i v application of small amounts of angiotensin strengthens the dipsogenic and ADH releasing effects of an infusion of hypertonic saline into the 3rd brain ventricle If this is the case it would indicate that the observed sodium angiotensin synergism is of physiological importance in the regulation of water metabolism

It is generally agreed that salt loading in most species is accompanied by an expansion of the extracellular fluid volume and an elevation of the glomerular filtration rate which facilitates renal sodium excretion However the Na reabsorption in the proximal tubule of the kidney may also become depressed under these circumstances (Dirks Cirksena and Berliner 1965) It has been suggested that this depression is effected by a humoral agent acting as a natriuretic hormone Infusions of hypertonic saline into the 3rd ventricle have been found to elicit conspicuous natriuresis in goats maintained on a salt supplemented diet (Andersson *et al* 1969 b) It indicates that the hypothalamus or adjacent parts of the brain may participate in the regulation of renal Na excretion The experiments performed on goats in normal water balance during the present study give no clear indication of a conjoint action of angiotensin and hypertonic NaCl on a brain mechanism which facilitates renal Na excretion The natriuretic effect of the combined intraventricular infusions was in most cases greater than that obtained by similar infusions of angiotensin alone or of merely hypertonic saline However the increased Na excretion seen during i v control infusions in 2 of the goats leaves the possibility open that angiotensin may have been transferred from the cerebrospinal fluid to the systemic circulation to such an extent that its vascular or renal effects contributed to increase the sodium excretion The situation was different in the hydrated animal Here the intraventricular infusion of angiotensin alone produced less increase in renal Na excretion than

in the not hydrated animal. In contrast, the infusions of angiotensin together with hypertonic NaCl induced extreme natriuresis of long duration. It indicates that angiotensin makes neurons involved in a central control of salt balance more sensitive to an elevated extracellular Na^+ concentration, maybe by facilitating the transport of Na^+ into these neurons. Then, the fact that hydration was needed to reveal a clear sodium-angiotensin synergism and to obtain maximum natriuresis in response to the combined infusions, implies that both an elevated intracellular Na^+ concentration and an expanded fluid volume are necessary for an optimal activation of a brain mechanism which stimulates renal salt excretion.

Sealey, Kirshman and Laragh have recently (1969) provided strong evidence for the idea that a natriuretic hormone exists, and that it is involved in the day to day physiological regulation of sodium balance. They have demonstrated a substance in plasma and urine from man and sheep which produces marked increases in the rate of Na excretion of appropriately volume expanded assay animals. This substance is not detectable in sodium-depleted subjects but consistently appears in sodium loaded subjects. The hormonal agent may not act immediately and its activity can be apparent for up to 3 hrs. In the goat the centrally induced natriuresis developed gradually, reached maximum after the 30 min intraventricular infusions, and persisted for up to 3 hrs. This time course indicates that the effect on the kidney may have been mediated by a humoral agent which is identical with the natriuretic substance demonstrated by Sealey *et al.* (1969). This would explain also why the greatest natriuretic effect was obtained in volume expanded goats.

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From the Psychological Laboratories, University of Stockholm, Sweden

The Diurnal Rhythm of Adrenaline Secretion in Subjects with Different Working Habits

By

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Abstract

PATKAI P. *The diurnal rhythm of adrenaline secretion in subjects with different working habits.* Acta physiol. scand. 1971. *81.* 30—34.

Subjects classified as habitual morning *vs* evening workers on the basis of their answers to a questionnaire were compared with regard to catecholamine excretion under conditions of relaxation in the morning and in the evening. It was shown that individuals who preferred to work in the evening excreted more adrenaline in the evening than in the morning, while individuals characterized by morning alertness excreted more adrenaline in the morning than in the evening, also when relaxing. With regard to noradrenaline excretion there was no marked difference between the groups.

The present investigation forms part of a larger project concerned with the relationship between catecholamine excretion and psychological functions. (Among the reviews in this field may be mentioned those of Euler 1967 and Frankenhaeuser 1970.) Earlier experiments (e.g. Pátkai *et al.* 1967, Frankenhaeuser *et al.* 1968, Frankenhaeuser and Rissler 1970) have shown a positive correlation between catecholamine levels, especially adrenaline level as measured by urinary excretion, and performance. These results were interpreted in the light of the hypothetical inverted U function relating behavioural efficiency to arousal level. According to this hypothesis efficiency increases as arousal increases to a medium level and then declines as arousal rises still further. It was assumed that adrenaline excretion would serve as an indicator of arousal level. Thus performance would be positively related to low to moderate adrenaline levels.

Additional information concerning the part played by adrenaline in the regulation of arousal was obtained in a study by Levi (1966), where the subjects were exposed to a 72 hr vigil under conditions which simulated combat. He found that adrenaline excretion, measured at three-hourly intervals, showed a regular sinus-shaped diurnal variation. The lowest excretion values were obtained just after midnight, coinciding with periods of maximum subjective fatigue and poorest performance.

The present study was undertaken to relate adrenaline excretion to individual differences in diurnal variations in alertness as reflected by working habits It is an everyday observation that some persons are habitually early risers, being more alert and efficient in the morning they prefer to work at that time of the day rather than late in the evening Others prefer to work late and often need a couple of hours in the morning before they can start working efficiently In this study these two types of individuals (called 'morning workers and 'evening workers) were compared with regard to their adrenaline excretion in the morning and in the evening It was expected that individuals who habitually preferred to work in the morning would excrete more adrenaline and feel more alert and efficient in the morning than in the evening The opposite tendency was expected for individuals characterized by evening alertness

Methods

Questionnaire used for selection of subjects

The subjects were chosen on the basis of their answers to a questionnaire constructed to obtain assessments of 'students working habits It comprised 21 questions about everyday routine Items with high face validity were chosen most of which might be roughly classified as belonging to one of three cate[illegible]
in the morning (2) in the [illegible]
The number of alternat ve [illegible]
were also asked to state (4 [illegible]
alertness vs sleepiness was [illegible]
were included in order to [illegible]
"evening' Thus they were for instance required to illustrate graphically how their level of general alertness varied during day and night

1	Do you usually a) get up as soon as your alarm clock rings or b) stay in bed for some time afterwards?	a)	b)		
		% 43.3	56 7		
2	Is it difficult for you to keep awake in the evenings?	Often	Sometimes	Seldom	
		% 5 5	35 8	58 7	
3	Which is your most efficient working time a) morning b) afternoon c) evening d) night?	a)	b)	c)	d)
		% 37 0	16 6	36 4	10 0
4	Are you a) alert in the morning and tired in the evening b) tired in the morning and alert in the evening c) alert both in the morning and in the evening d) tired both in the morning and in the evening? Denote the strength of your tendency with a number between 1 and 5 (1 strong tendency 3 as most persons 5 weak tendency)	a)	b)	c)	d)
		% 14 7	47 6	30 1	7 6

Answers were scored on a point basis a certain response being given a value of 1 indicating either morning or evening alertness A few answers chosen only by a small number of students were given additional weights e g the answer Often to question 2 above) Thus each student obtained two total scores one for morning alertness and one for evening alertness and students with the highest relative differences between the two scores were selected as subjects for the study Students with regular working times or parents of small children were not chosen

Subjects and procedure

The subjects were 19 students of psychology 7 males and 12 females They were selected from a group of 122 students Ten of the subjects were morning workers and 9 were "evening workers

Two urine samples were obtained from each subject, one at 9 a m and the other at 10 30 p m , following a period of 90 min quiet reading at home in a semi recumbent position in bed The subjects were also requested to estimate their alertness and their concentration ability during these two time periods Both sessions were carried out on the same day

Subjective alertness and ability to concentrate

After each session the subjects estimated the degree of subjective alertness and ability to concentrate during the session A graphic scale was used which had two endpoints, representing extreme values of the two variables The middle of the line marked the subject's normal alertness and concentration ability, respectively The ratings were expressed as distances (in cm) from that endpoint of the scale which denoted the lowest degree of each variable

Urinary catecholamines

Each subject was instructed to empty his bladder before each session, to collect urine at the end of the session and to adjust the pH of the sample to about 3 with 1 N HCl The equipment needed for taking care of the samples had been handed out in advance The subjects had been carefully informed about the conditions which might affect their catecholamine excretion [illegible] The urine volume [illegible] til analyzed by the [illegible]

Results

Subjective alertness and concentration ability

Fig 1 shows mean estimates of subjective alertness and concentration ability for the two groups during the two sessions The subject's normal value is represented by 7 5 and higher figures designate increased alertness and concentration ability Changes in the estimates were in the expected direction for both groups in both variables The differences between values in the evening and in the morning were significant only for evening workers (alertness $t = 14.38$ concentration ability $t = 11.20$ $p\ s < 0.001$) However the two groups were significantly different with regard to differences between their estimates in the morning and in the evening (alertness $t = 5.00$ concentration ability $t = 4.49$, $p\ s < 0.001$)

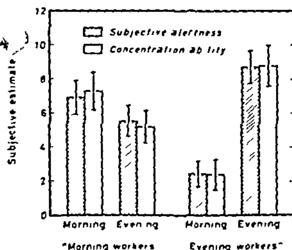


Fig 1 Subjective alertness and concentration ability in the morning and in the evening for "morning workers" and evening workers

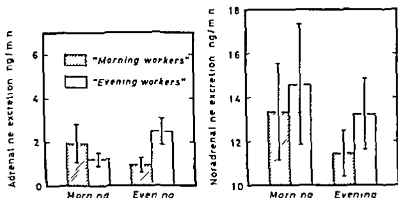


Fig 2 Means and standard errors for adrenaline and noradrenaline excretion in the morning and in the evening for "morning workers" and "evening workers" (Mean excretion time varied between 90 6 and 95 9 min)

These results indicate that the questionnaire served as a satisfactory tool for selecting subjects representing opposite extremes with regard to diurnal alertness patterns

Catecholamine excretion

Fig 2 shows mean adrenaline and noradrenaline excretion in the morning and in the evening for the two groups of subjects It is seen that 'morning workers' tend to excrete more adrenaline in the morning than in the evening while the opposite trend holds for "evening workers' The difference between the two adrenaline excretion values within each of the groups was significant only for 'evening workers' ($t = 2.78$, $p < 0.05$) Again, when the two groups of subjects were compared with regard to excretion values in the evening and in the morning significant differences in adrenaline excretion were found ($t = 2.98$, $p < 0.01$) Noradrenaline excretion showed no consistent differences

The excretion values of both catecholamines were rather low compared to low activity values obtained in other investigations (*e g* review by Frankenhaeuser 1970) This discrepancy, however is likely to be associated with the fact that in previous studies the control sessions have taken place in the laboratory, thus involving some degree of anticipation In this study, however, subjects were relaxing at home and *they were also allowed to choose the kind of literature (fiction or newspapers) they* wanted to read Seasonal variations in hormone activity have probably also influenced the results Thus, in the two recent studies (Feller and Hale 1964, Johansson Frankenhaeuser and Lambert 1969) it was shown that catecholamine excretion was significantly lower during spring than during winter Since this study was carried out during the second half of May (mean outdoor temperature on days of urine collection was 12 3° C) it is possible that the relatively low rate of catecholamine-excretion is associated with the time of the year

The results of the present investigation are consistent with the view that adrenaline release is closely related to arousal level Furthermore, the data support the hypothesis that intraindividual differences in diurnal variations in alertness and adrenaline secretion follow the same trend and may, hence, be determined by one and the same underlying factor It should be noted that both groups were examined under relaxed conditions Thus, the differences obtained in adrenaline excretion are not due to differences in overt activity

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From the Psychological Laboratories University of Stockholm, Sweden

Interindividual Differences in Diurnal Variations in Alertness, Performance, and Adrenaline Excretion

By

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Abstract

PATKAI, P *Interindividual differences in diurnal variations in alertness, performance, and adrenaline excretion* Acta physiol scand 1971 *81* 35—46

Subjects classified as habitual morning vs evening workers on the basis of their answers to a questionnaire, were compared with regard to day time variations in catecholamine excretion and performance Adrenaline excretion in morning workers was highest in the morning and decreased gradually during the day, while evening workers showed nearly constant excretion values The performance of morning workers did not vary during the day, while evening workers showed a steady improvement performing best in the evening In addition a significant difference between the two groups was found in the personality dimension of extraversion introversion showing that evening workers were more extravert and morning workers more introvert The constancy of individual diurnal rhythms and their relations to personality traits, are discussed

In a previous study (Patkai 1970) catecholamine excretion in the morning and in the evening was examined in subjects characterized by morning vs evening alertness (called 'morning workers' and evening workers") Under relaxed conditions, evening workers were shown to excrete more adrenaline in the evening than in the morning, while the opposite trend held for "morning workers' These results were interpreted as consistent with the view that adrenaline release is closely related to arousal level This interpretation is in line with the results of several investigations (*e g* Patkai *et al* 1967, Frankenhaeuser and Rissler 1970, Patkai 1970 a)

The present investigation is a continuation of the previous study Again, two groups of subjects were used, characterized by morning vs evening alertness

The purpose of the investigation was threefold

1 to check the results of the first study on a new sample of subjects studied under different conditions (not relaxing)

2 to study changes in the performance of concentration tasks in relation to changes in subjective alertness and adrenaline excretion

3 to explore possible differences in personality variables between the two groups of subjects with different working habits

Methods

Subjects and general design

The subjects were 22 female students of psychology Their ages ranged between 19 and 43 years (mean 23.3) These subjects were chosen with the aid of the same questionnaire which was used in the first study (Pátkai 1970), where it proved to be a satisfactory tool for selecting subjects representing opposite extremes with regard to diurnal alertness pattern It was given to a group of 186 students, from which 22 subjects, 11 "morning workers" and 11 "evening workers" were selected for investigation

Each subject participated in one whole-day session, lasting from 7 30 a m. to 10 00 p m Performance in the concentration tasks was measured early in the morning, in the middle of the day, and late in the evening Subjective estimates of sleepiness, concentration ability, and mood were given by the subjects nine times during the experiment Five urine samples were collected for assessment of urinary catecholamines

Working habits and variations in general alertness

In a semi-standardized interview the subjects were asked about their working habits since how long during their lives had they felt alert in the morning or in the evening, respectively, how many times had they changed their diurnal working habits and why, had they been subjected to any influences which ought to have changed their habits but did not, what working habits did members of their family have, etc. The main purpose of the interview was to obtain some information about the constancy of the subjects' working habits, *i e* how resistant they were *to environmental influence*

As a check on the assumption that differences in working habits reflect differences in diurnal variations in alertness the subjects were asked to rate subjective sleepiness and ability to concentrate A graphic scale was used which had two endpoints, representing extreme values of the two variables The middle of the line marked the subject's normal alertness and concentration ability, respectively The ratings were expressed as distances (in cm) from that end point of the scale which denoted the lowest degree of each variable

To assess possible changes in mood, an adjective list was employed which contained 22 adjectives The subject was instructed to mark those adjectives which described best how she felt at that moment

Dependent variables

Performance tests Three performance tests were used a reaction time test an arithmetic test and a colour word test Since earlier investigations *e g* Freeman 1940 Kleitman 1963, Sjöberg 1968 have shown that reaction time measures are especially sensitive to changes in arousal the reaction time test was considered the main instrument for assessing performance changes It was administered three times during the day in the morning in the middle of the day and in the evening The other two tests were administered during the two main test sessions only in the morning and in the evening in order to occupy the subjects during a relatively long time required for the collection of urine samples

In the reaction time test three light bulbs of different colours were used as stimuli The subject was instructed to react to only one of them by pressing a key with her right hand The stimuli were presented manually in three 5 min trials without pause Each trial contained 75 stimuli in random order 25 of which were to be reacted to Before the very first trial each subject had about 1 min of practice Reaction time (in msec were recorded as well as the number of omissions and wrong reactions

The arithmetic test constructed by Norinder unpubl consists of arithmetic problems arranged in squares each square containing two rows of digits There are two digits in each row which have to be either added or subtracted If this operation results in a larger number in the first row the second row is to be subtracted from it In the opposite case the two rows are to be added The result of the second operation is noted as the solution of the task The number of items correctly solved during 15 min and the number of errors were recorded

In the colour word test one part Card C of the Stroop test Thurstone and Mellinger 1953) was used This consists of 100 colour words which are printed in different colours on a gray background in random order The combination of words and colours is incongruent so that the word yellow for example may be coloured blue etc The subject's task is to ignore the word and name the colour of the print as quickly as possible The time needed for completing the task and the number of errors were recorded

Urinary catecholamines Urine samples were collected by voluntary voiding five times during the experiment, at 9 30 a m 11 00 a m 1 00 p m 8 30 p.m and at 10 00 p.m The urinary volume was measured and pH adjusted to about 3 by addition of 2 N HCl Samples were then stored at −18° C until analyzed by the fluorimetric technique of Euler and Lishajko 1961

The subjects had been carefully informed about the conditions which might affect their catecholamine excretion and which had to be avoided Smokers (6 subjects, 2 'morning workers' and 4 'evening workers', none of whom was a heavy smoker) were allowed to smoke one to

1956)

Procedure

The experimental procedure was exactly the same for all subjects The following time scheme describes the subjects' activities during the five time periods from which urine samples were obtained It should be mentioned that in the laboratory, the subjects were seated in an arm chair during the whole experiment

7 30 a.m.—9 00 a m two test sessions, each consisting of a 15 min reaction time test, a 15 min arithmetic test, and the colour word test administered twice (For half of the subjects

tive estimates and

atory During this

probably stressful efffects of the alternative procedure of keeping the subjects in the laboratory for 14 5 hrs It was hoped that the experiment would in this way more resemble a normal working day Care was also taken to avoid efffects of anticipation by informing the subjects in advance of the general procedure and the purpose of the study

Results

The constancy of the subjects' working habits

All the subjects but two "morning workers" stated that they had always been "morning workers" or 'evening workers", respectively These two subjects had originally been, as they put it, "normal day-workers" but they gradually became "morning workers" during their school-time School experiences failed, however, to change the habits of "evening workers" who often reported that they had had difficulties both at home and in school because of their late habits Several of them seemed to have a bad conscience about their being "evening workers", they chose morning groups at the university, for instance, or tried in some other way to force themselves to get up early On the other hand, "morning workers" often pointed out that they never caused their mothers any trouble at bedtime

The most effective time for intellectual work, as stated by the subjects, occurred between 8 and 12 a m for "morning workers', and between 9 and 12 p m for evening workers'

TABLE I Means and standard errors for subjective sleepiness and concentration ability during five time periods

	Time period									
	7.30 a.m.—9.00 a.m.		9.00 a.m.—11.00 a.m.		11.00 a.m.—1.00 p.m.		6.30 p.m.—8.30 p.m.		8.30 p.m.—10.00 p.m.	
	M	SE	M	SE	M	SE	M	SE	M	SE
Morning workers										
Sleepiness	7.9	0.7	7.4	0.8	9.0	0.6	6.4	0.7	4.6	0.8
Concentration	7.8	0.6	7.9	0.6	8.9	0.5	7.1	0.6	5.6	0.8
Evening workers										
Sleepiness	3.9	0.6	4.8	0.6	8.3	0.7	8.3	0.8	8.6	0.6
Concentration	3.9	0.6	5.5	0.6	8.4	0.6	8.5	0.8	8.7	0.5

Subjective variables

Sleepiness and concentration ability Means and standard errors of subjective sleepiness and concentration ability for the two groups are presented in Table I. The subject's normal value is represented by 7.5 and higher figures designate increased alertness and concentration ability. During the first and last time periods the subjects gave 3 estimates, the means of which are presented in the table.

As expected, the two groups showed distinctly different time patterns in both variables, as also indicated by the significant interactions between groups and time periods ($F = 13.10$, $df = 4/80$, $p < 0.001$ for sleepiness and $F = 12{,}58$, $df = 4/80$, $p < 0.001$ for concentration ability). Two-way analyses of variance (Table II) showed that the differences between time periods were significant for both groups. Morning workers felt significantly more alert and efficient during the first two time periods as compared with the last two time periods in the evening (p varied between 0.05 and 0.001). Evening workers, on the other hand, gave significantly higher estimates of subjective alertness ($p < 0.01$) and efficiency ($p < 0.001$) during the last three time periods as compared to the first two conditions.

Changes in subjective estimates during the day were highly similar for all subjects within each group. This group homogeneity was borne out by the fact that inter-individual differences did not yield a significant F-quotient (Subjects in Table II), which is the usual case especially when subjective estimates are analyzed.

These results indicate the usefulness of the questionnaire for selecting subjects representing opposite extremes with regard to diurnal alertness patterns.

Mood variations and their relations to other variables As a crude check of possible changes in mood during the day, the frequency of every adjective marked at different times was computed for each individual in each group. Distinct differences between the patterns of fr[illegible] distributions for the two groups of subjects were found in the following var[illegible]: cheerful, amused, brisk, tired, efficient and drows[illegible]. Morning workers tended to describe their feelings in more positive terms

TABLE II Summary of analyses of variance for catecholamine excretion and urine volume in two groups of subjects with different working habits

Source	Morning workers				Evening workers			
	SS	df	MS	F	SS	df	MS	F
	Adrenaline excretion							
Time periods	66 95	4	16 74	6 79***	17 74	4	4 43	1 40
Subjects	61 19	10	6 12	2 47*	67 77	10	6 78	2 14 *
Interaction	99 34	40	2 48		126 98	40	3 17	
Total	227 48	54			212 49	54		
	Noradrenaline excretion							
Time periods	1286 62	4	321 66	16 08 ***	878 16	4	219 54	4 18
Subjects	1823 83	10	182 38	9 12***	2914 90	10	291 49	5 55***
Interaction	799 92	40	20 00		2099 12	40	52 48	
Total	3910 37	54			5892 18	54		
	Subjective sleepiness							
Time periods	121 94	4	30 49	6 69	224 86	4	56 22	12 49***
Subjects	87 85	10	8 79	1 93	69 64	10	6 96	1 55
Interaction	182 29	40	4 56		180 15	40	4 50	
Total	392 08	54			474 65	54		
	Concentration ability							
Time periods	66 19	4	16 55	5 03**	208 87	4	52 22	14 39***
Subjects	77 61	10	7 76	2 36	62 68	10	6 27	1 73
Interaction	131 42	40	3 29		145 18	40	3 63	
Total	275 22	54			416 73	54		

* p < 0 05, ** p < 0 01, *** p < 0 001

in the morning than in the evening No differences between the groups were found in the variables 'indifferent', "expectant' and "relaxed', while some adjectives describing negative feelings, ('gloomy", 'morose' and 'dispirited) were only marked by evening workers' in the morning

Marked interindividual differences were noticed in the subjects overall tendencies to choose more negative or more positive adjectives possibly reflecting their attitudes to the whole experimental situation In order to check this possible tendency and its consequences individual 'attitude scores' were computed on the basis of subjects' choice of those adjectives which were assumed to reflect positive or negative attitudes (as estimated by two judges) 'Attitude scores' were found to be related to scores on the Social Desirability Scale individuals with high scores on the scale were significantly more positive ($t=2\ 11$ $df=20$ $p<0\ 05$) than were subjects with low scores This suggests that subjects with high positive attitude scores might be reluctant to report any possible negative feelings

Catecholamine excretion

Fig 1 shows the time patterns of adrenaline excretion for the two groups of subjects It is seen that adrenaline excretion in morning workers was highest in the morning and decreased at a relatively steady rate during the day 'Evening workers" th

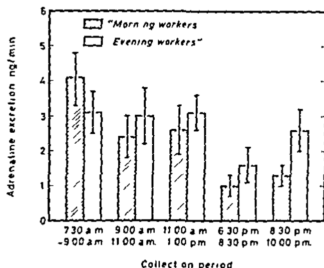


Fig 1 Diurnal pattern of adrenaline excretion (means and standard errors) in two groups of subjects with different working habits

other hand showed nearly constant excretion values during the whole experimental day so that compared to "morning workers" they excreted somewhat less adrenaline in the morning and more in the evening As in the first study, the two groups differed significantly with regard to the difference between their adrenaline excretion in the morning and in the evening ($t = 2\,00$ $df = 20$ $p < 0\,05$, one-tailed) The interaction between groups and the five time periods was however not significant (3 way classification $F = 1\,47$ $df = 4/80$ NS)

Analyses of variance computed separately for the two groups (Table II showed that the differences in adrenaline values between the time periods were significant only for "morning workers" t tests showed that adrenaline excretion was significantly higher during the first time period than during the other four periods (p varied between 0 05 and 0 001) The differences between the 2nd and 4th and the 3rd and 4th time periods were also significant ($p < 0\,05$)

Fig 2 shows that the highest values of *noradrenaline* occurred between 11 a m and 1 p m in both groups Although "evening workers" had somewhat higher values during the whole day especially in the morning and in the evening the difference between the two groups was not significant (a non significant difference of the same direction was also found in the first study) Differences between time periods were significant for both groups (Table II) t tests showed that periods 2 and 3 for "morning workers" differed significantly from each other and from the other periods (p varied between 0 05 and 0 001 For "evening workers" the 1st and 3rd periods differed significantly from the last two periods (p between 0 05 and 0 01)

While noradrenaline values were in good agreement with the values obtained in previous investigations (e g review by Frankenhaeuser 1970) adrenaline excretion was rather low in both groups of subjects It should be noted that this study was

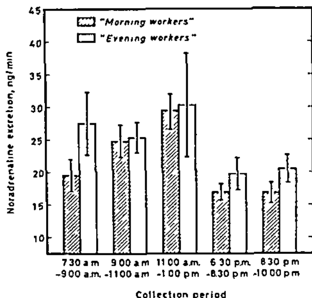


Fig 2 Diurnal pattern of noradrenaline excretion (means and standard errors) in two groups of subjects with different working habits

carried out in May and June (mean outdoor temperature on days of urine collection was 13 0° C) Since adrenaline excretion seems to be sensitive to seasonal variations (Johansson, Frankenhaeuser and Lambert 1969) it is possible that this factor influenced adrenaline excretion in a selective manner

Performance

Means and standard errors for performance scores of the two groups are shown in Table III As mentioned above (p 2), reaction times were measured three times during the day, while the colour-word test and the arithmetic test were only administered in the morning and in the evening

In the reaction time test, the two groups of subjects had distinctly different time patterns (Fig 3) While "morning workers" performed on approximately the same level on all three occasions, "evening workers" showed a steady decrease in their time scores, so that they performed best in the evening The difference in pattern between the groups was borne out by a significant interaction term for groups × time periods in a three-way analysis of variance (Table IV) Analyses of variance and t tests, computed separately for the groups, showed that differences between the three time periods were significant only for "evening workers" ($p < 0.02$ and 0.001) In the error scores no differences between the groups were found (Table IV) indicating that both groups probably followed the same strategy (not working too fast at the expense of accuracy)

TABLE III Means and standard errors for performance scores in two groups of subjects with different working habits

Performance	Morning workers						Evening workers					
	Morning		Noon		Evening		Morning		Noon		Evening	
	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE
Reaction time, msec	456	2 9	437	3 3	447	2 8	452	1 6	381	1 0	345	0 9
Reaction time no of errors	3 1	0 9	2 3	0 7	2 5	0 6	4 9	1 8	2 1	0 5	4 1	0 6
Colour word test, sec	1 5	0 1			1 3	0 1	1 5	0 1			1 3	0 1
Colour word test, no of errors	6 8	1 5			3 1	0 5	7 5	1 1			5 9	1 1
Arithmetic test, no of correct responses	256 7	11 6			272 2	15 8	224 2	21 7			259 5	28 1
Arithmetic test, no of errors	10 6	1 6			7 0	1 0	15 3	3,1			10 7	1 6

In interpreting the absolute changes in reaction speed, practice effects should also be taken into consideration If this factor could have been kept under control the *performance of morning workers would probably have been still poorer in the* evening and evening workers would have performed less well Thus the similarity between changes in adrenaline excretion and in performance would have been still more clearcut.

In the colour word test and the arithmetic test both groups performed better in the evening than in the morning (Table III) The improvement in the colour word test was significant for both groups ($p<0.02$ for evening workers and $p<0.01$ for morning workers) but in the arithmetic test only evening workers were significantly better in the evening ($t=3.14$ df $=10$ $p<0.02$)

TABLE IV Summary for analyses of variance for reaction time and reaction time errors

Source	Reaction time				Errors			
	SS	df	MS	F	SS	df	MS	F
Groups	[illegible]	1	[illegible]	3 13	[illegible]	1	[illegible]	1 29
Conditions	[illegible]	2	199 82	[illegible]***	[illegible]	2	18 43	[illegible]
Groups x Conditions	[illegible]	2	[illegible]	[illegible]***	13 42	2	6 71	1 0
Subjects	[illegible]	20	[illegible]	15 48***	[illegible]	20	15 27	2 19
Remainder	[illegible]	[illegible]	10 03		[illegible]	40	[illegible]	
Total	[illegible]	[illegible]			[illegible]	63		

*** $p<0.001$

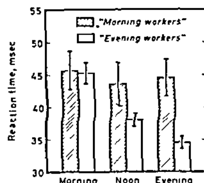


Fig 3 Means for reaction time measurements at different times of the day in two groups of subjects with different working habits

TABLE V Scores in personality questionnaires for morning workers and evening workers

Questionnaire	Morning workers M	Morning workers SE	Evening workers M	Evening workers SE
Psychasthenia Scale	21 45	2 29	15 91	1 92
Social Desirability Scale	8 55	1 41	8 64	1 36
Extraversion Scale	11 91	1 24	16 18	0 95
Neuroticism Scale	10 91	1 36	9 73	1 34
Manifest Anxiety Scale	8 00	1 60	6 73	1 11
Lie Scale	3 18	0 70	3 09	0 74

Personality variables

Table V shows means and standard errors of questionnaire scores for the two groups of subjects Compared with "morning workers", "evening workers" had significantly higher scores on the Extraversion Scale ($t=2\ 72$, $df=20$, $p<0\ 02$) and somewhat lower scores on the Psychasthenia Scale ($t=1\ 85$ $df=20$ $p<0\ 10$) No differences between the groups were found in scores on the other questionnaires

Discussion

Diurnal variations in adrenaline excretion and alertness

The results of the present investigation lend further support to the hypothesis about a close relation between adrenaline excretion and alertness Subjects with different working habits were shown to have different patterns of adrenaline excretion during day time Thus, adrenaline excretion in "morning workers" was highest in the morning and decreased steadily during the day On the other hand "evening workers" displayed about the same excretion level during the whole experimental period although it was expected partly on the basis of the previous study that they would excrete more adrenaline in the evening than in the morning Several factors may have contributed to the fact that this was not the case The great majority of "evening

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Acta physiol scand 1971. 81. 47—53

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Effect of Prolonged Chloralose Anesthesia on Acid-Base Balance and Cardiovascular Functions in Dogs

By

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Abstract

ARFORS, K E, G ARTURSON and P MALMBERG *Effect of prolonged chloralose anesthesia on acid base balance and cardiovascular functions in dogs* Acta physiol scand 1971 *81* 47—53

Dogs under light even α-chloralose anesthesia of 6 hrs duration showed no time dependent effect of anesthesia on mean arterial pressure, cardiac index and oxygen consumption The Pa_{CO_2} remained stable as did respiratory rate but base excess decreased moderately and heart rate increased Compared with a group of conscious dogs Pa_{CO_2} and mean arterial pressure were slightly increased

Chloralose anesthesia has been used in experimental work since the beginning of the century and in recent years it has been shown that α-chloralose anesthesia might have advantages in some types of investigations Thus van Citters *et al* (1964) recommended α chloralose in studies on the central neural regulation of the cardio vascular system since reflex activity is better maintained Furthermore, α chloralose had less effect on basal cardiac performance than pentobarbital anesthesia The aim of the present investigation was to evaluate the time dependent effect of carefully controlled light chloralose anesthesia on acid base balance and cardiovascular functions in dogs For comparison the same parameters were investigated in trained conscious dogs Furthermore the effect of polyethylenglycol (PEG) and borate used as solvents for the chloralose were compared

Material and Methods

27 healthy dogs of the Vorsteh type aged 7—9 months weight 21 7 2 6[1] and of the same breed were used

Arterial pH was measured with a microcapillary electrode (Radiometer Copenhagen) Pa_{CO_2} and base excess were calculated from the Siggaard Andersen curve nomogram (Siggaard Andersen 1962) by measuring pH after equilibration with two different gas mixtures in

[1] Mean standard deviation

means of an infra red heating lamp according to the oesophageal temperature The operating table was kept at 30° C

were disconnected from the spirometer and breathed room air spontaneously

B *Additional studies*

Five dogs with indwelling aortic catheters were anesthetised according to the scheme outlined in the main study Arterial pressure, heart rate and acid base balance were measured before anesthesia and then continuously for 6 hrs No additional fluid was given in this group

15 dogs were anesthetized with α chloralose 4 % in 5 % borate (25 mg/kg) and sodium pentobarbital (15 mg per kg) After induction of anesthesia a continuous infusion of 4 % chloralose in 5 % borate was started 7 of these dogs received i v saline at a rate of 3 ml/kg/hr In these 15 animals less emphasis was put on maintaining an even depth of anesthesia Acid base status oxygen consumption arterial pressure, heart rate respiratory rate and rectal temperature were measured All dogs recovered from anesthesia and were followed for several days

Results

I *Conscious dogs*

In 11 dogs acid base balance, arterial pressure and heart rate was measured The results of the first of these measurements in each dog are shown in Table I and Fig 1 In 5 of the dogs acid base measurements were also made every hour for 6 hrs and once a day for 6 days Analysis of variance using a one way lay-out was used to test *differences in acid base values between conscious dogs measured every hour for 6* hrs and once a day for 5 days respectively In most cases significant differences in measured parameters were found between dogs, see Table II Estimate of S D within dogs and between the means of different dogs shows that these are of similar size

II *Chloralose anesthesia for 6 hrs*

A *Main study* (Fig 1 and 2 and Table I)

After induction of anesthesia with *chloralose in PEG* the mean value of Pa_{CO_2} was slightly higher than in the group of conscious dogs but the difference was not signifi

3		4		5		6	
Mean	S D	Mean	S D	Mean	S D	Mean	S D
7 36	0 04	7 36	0 05	7 35	0 07	7 33	0 05
36 5	6 1	35 1	4 7	35 4	5 2	36 6	7 4
—4 6	2 9	—5 1	2 9	—5 7	2 5	—6 2	2 2
23	10	22	9	23	11	22	10
130	9 0	136	8 5	133	6 6	128	10
127	17 9	127	20 5	137	23 3	144	23 8
156	39 8	155	60 7	159	49 9	168	38 6
7 0	0 8	7 4	1 2	7 4	1 1	7 1	0 8
38 4	0 5	38 4	0 5	38 6	0 6	38 5	0 5

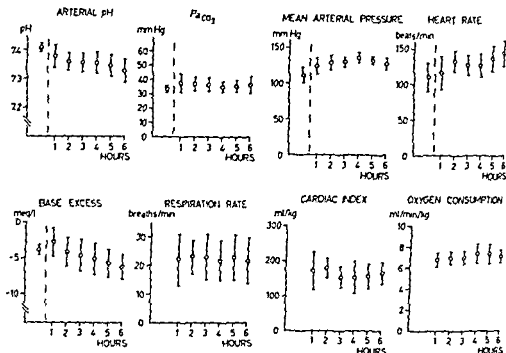


Fig. 1. Mean values ±95 % confidence limits from 11 conscious dogs (left of stippled line) and from 9 dogs in chloralose anesthesia.

cant. Pa_{CO_2} remained stable during anesthesia. The base excess slowly decreased during anesthesia (P [1—6 h][1] = 0.005). The arterial pH also tended to decrease but not significantly (P [1—6 h][1] = 0.06).

TABLE II

Dogs measured every hour for 6 hours	p-value when testing the hypothesis $\sigma_a = 0$	Estimates of σ_a	σ_e
pH	<0.001	0.017	0.019
Pa_{CO_2}	<0.001	1.93	2.22
BE	<0.0005	1.32	0.83
Dogs measured every day for 5 days			
pH	>0.3	0.005	0.022
Pa_{CO_2}	>0.05	1.72	3.04
BE	<0.05	0.84	1.33

Result of analysis of variance of acid base values in conscious dogs.
σ_a = S.D. for true dog means.
σ_e = S.D. for repeated measurements of one dog.

[1] P-value for the difference between the 1 hr and the 6 hrs determination.

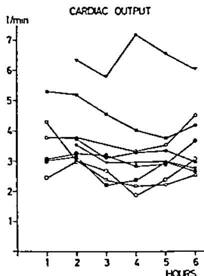


Fig 2 Individual values of cardiac output from 9 dogs in chloralose anesthesia

The mean arterial pressure was higher than in the conscious group of dogs ($P = 0.04$) 1 hr after induction of anesthesia and remained relatively stable The heart rate increased during anesthesia ($P\ [1—6\ h]^1 = 0.04$) No significant change in cardiac index or oxygen consumption was observed during anesthesia The average rate of chloralose infusion was 30 mg/kg/hr A marked diuresis was noted (4 ml/kg(hr)

B *Additional studies*

In 15 dogs anesthetised with *chloralose in borate* the mean values of acid base balance, oxygen consumption, arterial pressure and heart rate were not significantly different from the values in the main study (In this group the depth of the anesthesia varied In deep anesthesia there was a tendency towards increased Pa_{CO_2}, decreased respiratory rate and decreased arterial blood pressure) The average rate of chloralose infusion was 20 mg/kg/hr

Five dogs with an indwelling aortic catheter anesthetised with *chloralose in PEG* did not show any significant difference in acid-base balance or heart rate from the results in the main study In these dogs the arterial pressure measured continuously before and during anesthesia showed a tendency towards lower values 20 minutes after induction of anesthesia However, 1 hr after anesthesia arterial pressure had increased to higher values than before anesthesia Pa_{CO_2} increased after anesthesia The mean difference between Pa_{CO_2} before anesthesia and Pa_{CO_2} 1 hr after anesthesia was significant ($P<0.05$) (Student s t test for paired observations) The average rate of chloralose infusion was about 30 mg/kg/hr The urine flow rate was 4 ml/kg/hr

Discussion

Although it would be preferable to use conscious, trained animals in cardiovascular studies (Rushmer *et al.* 1959, Pirano *et al.* 1969) much research work must be done under general anesthesia, *e.g.* using pentobarbital or chloralose. Unfortunately pentobarbital alters some hemodynamic functions and making it difficult to maintain steady state conditions (Linegar *et al.* 1936, Myers *et al.* 1954, Page *et al.* 1954, Nash *et al.* 1956, Howell *et al.* 1959, Shabetai *et al.* 1963, Pirano *et al.* 1969). Many investigators claim that chloralose has less undesirable effects on cardiovascular function (Shabetai *et al.* 1963, van Citters *et al.* 1964). *There are, however, very few* reports on the effects of prolonged administration of chloralose.

In the present investigation special attention was paid to the animal material. All animals used were of the same type (Vorsteh) and of similar body weight. The small standard deviation obtained in the conscious dogs as shown further illustrates the homogeneity of the material (Table I and II).

Since chloralose is difficult to dissolve in water, PEG or borate were used as solvents. PEG is considered to be inert apart from acting as an osmotic diuretic while borate is toxic in high doses. LD_{50} for dogs (oral dose of borate) is 1 g/kg b.w. *(Flury 1956). In the present study borate given in a dose of 110 mg/kg did not* influence the parameters investigated. However, conscious dogs given borate in a dose of 450 mg/kg i.v. vomited. For this reason, in the main study, borate was used only to dissolve the chloralose used for induction of anesthesia.

The effect on respiration and cardiovascular functions of chloralose anesthesia depend on the level of anesthesia (Koppermann 1955, Balis and Monroe 1964, Mauck *et al.* 1965). This might explain some of the differences in results in the studies of Mauck *et al.* 1961, van Citters *et al.* 1964, and Bass and Buckley 1966. In the main study of the present investigation the rate of infusion of chloralose was *varied to keep an even and light depth of anesthesia according to Koppermann et al.* (1955) *i.e.* without spontaneous movements or response to painful stimuli, with increased tendon reflexes and with reactions to sound stimuli.

A slight decrease in arterial pH and base excess concomitant with a constant slight elevation in Pa_{CO_2} was found during chloralose anesthesia. This moderate metabolic acidosis could hardly be caused by the solvents used. Borate given to conscious dogs in high doses did not change arterial pH or base excess. (Own observations.) Furthermore, no significant differences in acid base balance were found in dogs anesthetised with chloralose dissolved in borate and in PEG respectively. It should be pointed out that the changes in acid base balance during chloralose anesthesia are moderate and might be of importance only if the duration of anesthesia is prolonged.

In our experience a rise in Pa_{CO_2} and a decrease in respiratory rate are early signs of increased depth of chloralose anesthesia. This is compatible with the decreased respiratory response to carbon dioxide found by *Dripps and Dumke (1943)*. The constancy of P[illegible] and respiratory rate obtained in the main study of the present investigation *is thus further evidence of an* even level of [illegible] th[illegible] oc[illegible] he experimental peri[illegible]d.

Cardiac output varied considerably in some dogs (Fig 2), but the mean values were essentially unchanged The variations in cardiac output in individual dogs in chloralose anesthesia should be borne in mind when interpreting results of cardiovascular experiments

The present results show that light chloralose anesthesia does change the values of some parameters compared to the conscious state (Pa_{CO_2} arterial pH, arterial pressure) However, they remain relatively unchanged during 6 hrs of anesthesia apart from a slight decrease in base excess (and pH) and a moderate increase in heart rate The dissolving agents used both have unwanted effects and need be used only when excessive fluid administration is considered a disadvantage

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Transient Dynamics of Ventilation and Heart Rate with Step Changes in Work Load from Different Load Levels

By

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Abstract

BROMAN SVETLANA and O WIGERTZ *Transient dynamics of ventilation and heart rate with step changes in work load from different load levels* Acta physiol scand 1971 *81* 54—74

Transient dynamics of ventilation (V) and heart rate (HR) in response to 650 kpm/min step changes in work load were analyzed in six male athletes performing submaximal cycling exercise in the supine position, with the steps initiated at different levels of stable state exercise, including "loadless" pedaling (0 kpm/min) By applying mathematical parameter identification, the responses of V to both positive and negative step changes in work load could be accurately described by first-order exponential models With the positive steps initiated at 0 300, and 650 kpm/min the means of the time constant estimates for V ranged from 67 to 101 sec, and seemed to be independent of the initial work level Shifting from rest to pedaling at zero load and back to rest caused abrupt changes in V whereas this was rarely the case after step changes from one work load to another with the subjects already pedaling The responses of HR required second-order models with the two time constants ranging from 9 0 to 11 7 sec and from 1 8 to 3 7 min and with the share of the slower component increasing with the initial work level Pure time delays were negligible indicating that the early readjustments in both V and HR are under neurogenic influence The results support the notion that in exercise the respiratory and circulatory control systems exhibit dissociated dynamic properties

In a recent study a systems analysis approach was used to analyze the dynamics of ventilation and heart rate in response to sinusoidal work load (Wigertz 1970) In the past, work loads with a rectangular wave form have been used extensively to determine stable state responses of ventilation and circulation under various conditions and more recently for evaluating their dynamic characteristics during the transient phases of exercise and recovery However no systematic quantitative analysis seems to have been carried out concerning the influence of the initial work level

In the present study an attempt was made to analyze the transient dynamics of ventilation and heart rate following positive and negative sustained step changes in

work load, initiated from different levels of stable state exercise This was done by fitting of mathematical functions to experimental data, utilizing a systems analysis approach and computer-oriented methods for parameter identification The experimental data were obtained from male athletes performing supine leg exercise on a cycle ergometer Since it was considered possible that the transition from motionless rest to exercise might constitute a special case, the transient behavior of the responses to abrupt shifts between rest and loadless pedaling were also examined

Methods

Six healthy male students participated in this study They were all well trained in endurance sports and engaged in similar training programs and physical activities Individual anthropometric and functional data are given in Table I

TABLE I Dimensional and functional data

Subject	Age (yr)	Weight (kg)	Height (cm)	Blood pressure supine (mm Hg)	$\dot{V}_{O_2 max}$* (l/min, STPD)
KK	23	64	166	140/85	4 0
LM	21	77	186	125/75	3 9
BN	22	76	180	145/95	5 5
JN	24	69	181	140/90	3 2
LP	23	88	192	130/85	4 9
KS	22	73	179	140/90	4 4

* From nomogram of Åstrand (1960)

Ergometer

All the experimental data were obtained with the subjects in the supine position, resting or performing leg exercise on an electrically braked cycle ergometer (Holmgren and Mattsson 1954), designed to keep the work load constant at varying pedaling rate

Recordings

Inspired minute volume ($\dot{V}$) was automatically and continuously computed breath by breath with a special purpose digital computer (Åstrom and Wigertz 1966) Input signals for the [illegible]

breath

Heart rate (HR) was obtained from chest electrodes and a linear cardiotachometer (Lindborg Ödman and Wigertz 1969) which gave an output signal directly proportional to the inverted time interval between successive R waves with its value held over the next heart period

For data processing purposes the V and HR signals of the 6 subjects were stored side by side on 12 of the 14 channels of an analog (FM) magnetic tape recorder (Ampex FR 100 C tape speed 1 7/8 ips) The remaining 2 channels were used for playback of prerecorded instructions for the experimenter (see Experimental procedure) and for recording of elapsed time to facilitate retrieval of signals from any given portion of the experimental protocol In addition an 8-channel ink recorder with rectilinear pen motion (Brush Mark 200 paper speed 12 mm/min) was used for monitoring of the signals during the experiments and for off line visualization of the time courses of signals reproduced from the magnetic tape The Brush recorder was supplemented by an X Y plotter (Moseley Model 7001 AM) to facilitate inspection of details in the tracings when desired

Experimental procedure

After being informed about the general course of the experiment excepting the sequence and

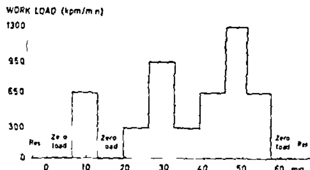


Fig 1 Experimental protocol presented as a work load time profile The protocol includes 3 rectangular work load waves of 650 kpm/min amplitude, starting from 3 different load levels 0 300 and 650 kpm/min Transitions between immobile resting and zero-load pedaling appear at start and end of protocol

magnitudes of the work loads the subject was positioned supine on a bed provided with a

verbal command with a 3 sec countdown the flywheel of the ergometer being kept turning to avoid the sudden effort of overcoming flywheel inertia All ensuing changes in work load were accomplished abruptly with the subject already pedaling This was done by remote control and without warning to avoid that work load changes could be anticipated by the subject The protocol included rectangular work load waves of 650 kpm/min amplitude starting and ending at three different load levels i.e. 0 300 and 650 kpm/min The subjects were re- … steps in work load were … 00 kpm/min which was … experiment the subject

… wo channels of the tape recorder Between experiments the tape was rewound and the recording shifted to two of the remaining channels The procedure was repeated until the signals from all six subjects had been …

Room temperature averaged 22 5° C and barometric pressure 753 mm Hg

Computations

To obtain individual stable state values for ventilation and heart rate the time averages of these variables over the 6th (5th) minute of each period of rest and exercise at different work levels were computed on line by using an integrating voltmeter

The transient dynamics of the ventilation and heart rate following the six positive and negative steps in work load were analyzed by digital computer techniques The methods used to accomplish this phase of the computations are described below (see Identification of dynamic parameters and App… … before the identification procedure could …

Individual transient response … eart rate were reproduced from th… … of a Hewlett Packard/Dymec A/D-conversion system including scanner and tape punch … odel DY 2010 B)

Mean transient responses The analog response signals for ventilation from all six subjects were simultaneously reproduced from the magnetic tape and continuously processed by use of an analog computer (PACE TR 48) to form a running mean of the group The heart rate data were treated similarly The signals were weighted to suppress the influence of inter individual amplitude differences on their mean dynamics For this purpose the input potentiometers of the summing amplifiers were adjusted according to the desired weight for each individual signal with the sum of all weights being equal to unity Individual weights were chosen so as to be inversely proportional to corresponding stable state response amplitudes The

output signal of the summing amplifiers thus gave the weighted means of the ensemble of heart rate and ventilation signals which for brevity will be referred to as the 'mean transient response" The output signal was digitized (1 sample/sec) in a manner similar to that used for the individual response signals

Reduction of noise and technical artefacts To eliminate technical artefacts such as dropouts

Identification of dynamic parameters

When the ventilatory and heart rate transient responses were visualized using the Brush recorder (*cf* Fig 2), or XY recorder, it became apparent that they resembled low order exponential time functions To define more accurately their time courses the transient responses were subjected to dynamic analysis by adopting the following first and second-order functions, $f_1(t)$ and $f_2(t)$, with the objective of estimating their unknown dynamic parameters

$$f_1(t) = a_0 + a_1 \exp[-(t - T_D)/\tau_1]$$
$$f_2(t) = a_0 + a_1 \exp[-(t - T_D)/\tau_1] + a_2 \exp[-(t - T_D)/\tau_2]$$

where a_0 a_1 and a_2 are amplitude coefficients, τ_1 and τ_2 time constants and T_D is pure time delay $f_1(t)$ and $f_2(t)$ are the solution curves to low-order linear differential equations for which the roots of the characteristic equations are real, distinct and negative when subjected to a step input forcing function

Whereas the first-order function was tried for both individual and mean transient responses the second-order function was tried only for the latter responses

made it possible to compute a measure of accuracy for all parameters estimates

Minimization of the value of a loss function was selected as a criterion of best fit The normalized loss function, hereafter termed L-value (see Appendix), permitted inter individual comparisons of best fits

An advantage of the present fitting procedure is that it can be carried out without knowledge of the final steady state values In the present study, the first 270 sec of the ventilatory tran s ents and the first 210 sec of the heart rate transients were processed These time segments had previously been tested separately for ventilation and heart rate and found to contain all the essential dynamic information

Results

Stable state values

The individual and mean stable state values at rest and at different levels of exercise are presented in Table II for ventilation and in Table III for heart rate Linear regression analysis showed that within the range 0 kpm/min (loadless pedaling) to 1300 kpm/min both ventilation and heart rate were linearly related to the work load in all subjects For the individual data the correlation coefficient (r) ranged from 0 964 to 0 997 for ventilation and from 0 989 to 0 997 for heart rate (r = 0 959 at the 1 % level) When calculated on the mean values (*cf* Tables II and III) the relationship between ventilation (V) and work load (WL) on the one hand and that between heart rate (HR) and work load on the other are expressed by the equations

V = 7 84 + 0 0376WL l/min   r = 0 980
HR = 78 5 + 0 0592WL beats/min   r = 0 995

TABLE II. Ventilation at rest and during the sixth minute of constant load supine leg exercise at zero load, 300, 650, 950 and 1 300 kpm/min *

Subject	Inspired minute volume (l/min ATP)					
	Rest	0 kpm/min	300 kpm/min	650 kpm/min	950 kpm/min	1 300** kpm/min
KK	5.2	10.0	16.4	29.8	41.2	65.2
LM	6.1	8.0	18.5	29.5	38.0	69.7
BN	5.9	10.4	15.9	23.4	32.1	46.7
JN	5.7	11.4	18.6	31.0	41.5	67.4
LP	8.7	14.2	22.2	32.4	42.3	55.8
KS	6.1	11.4	19.2	30.8	39.4	64.4
Mean	6.3	10.9	18.5	29.5	39.1	61.5

* Means of values obtained for work periods with identical load (cf. Fig. 1)
** Values refer to 5th min

TABLE III. Heart rate at rest and during the sixth minute of constant load supine leg exercise at zero load, 300, 650, 950 and 1 300 kpm/min *

Subject	Heart rate (beats/min)					
	Rest	0 kpm/min	300 kpm/min	650 kpm/min	950 kpm/min	1 300** kpm/min
KK	66	78	91	117	133	164
LM	70	87	101	124	136	170
BN	54	72	83	100	114	136
JN	73	95	110	136	153	183
LP	66	80	93	107	123	144
KS	62	75	89	111	127	156
Mean	65	81	95	116	131	159

* Means of values obtained for work periods with identical load (cf. Fig. 1)
** Values refer to 5th min

Transients in ventilation and heart rate following step changes in ergometric work load

Fig. 2 depicts the time courses of ventilation (left panel) and heart rate (right panel) in the six individuals resulting from a rectangular work load wave of 650 kpm/min amplitude and 6.5 min duration initiated from a work level of 300 kpm/min. The computed running means of the group are also shown in the figure. Fairly uniform response patterns ("on" and "off" transients) were observed for both ventilation and heart rate. However, whereas the ventilatory responses seem to follow a mono-exponential pattern, the heart rate responses show a fast initial phase followed by a slow adjustment to the final level, suggesting at least two exponential components. Similar time courses were observed with the rectangular work load waves initiated at the 0 and 650 kpm/min levels.

To estimate the dynamic parameters for the step responses of the two variables (see Methods, p. 57), first order fittings were applied to all the individual and mean

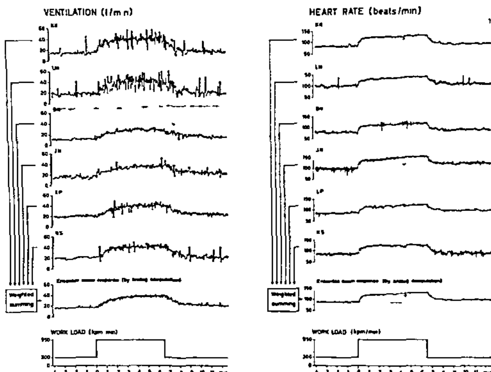


Fig 2 Individual and computed mean transient responses of ventilation (left panel) and heart rate (right panel) to positive and negative step changes in work load between 300 and 950 kpm/min The individual responses of each variable are shown as they appear on the Brush recorder when simultaneously reproduced from the magnetic tape together with the computed mean transient responses Note the reduction of noise in the computed curves

transient responses In applying a first-order model both for heart rate and ventilation, the time constants derived from such a model could be used not only for inter individual but also for intra-individual comparisons between the responses of the two variables

First order time constants The time constants (τ_1) and time delays (T_D) obtained with first order fittings to individual and mean transient responses for ventilation are given in Table IV (positive steps) and Table V (negative steps) The corresponding parameters for the heart-rate responses are given in Table VI and VII Also shown in the tables are the associated minimal L-values, the standard deviation for the time-constant estimates, as well as group means and standard errors for τ_1 and T_D

As can be seen in Table IV—VII, the inter-individual variations in the estimated time constants are much greater for ventilation than for heart rate However, it may be noted that the time constants computed from the mean transient responses of both variables differ but slightly from those calculated as arithmetic means This may be taken to indicate that any of these measures can be used to characterize the average" individual in the group The fact that the L-values are lower for the mean

TABLE IV First-order best fit parameters for ventilatory responses to positive steps in work load τ_1 = time constant, T_D = time delay, L = value of associated loss function

Subject	τ_1 (sec) 0—650 (kpm/min)	τ_1 (sec) 300—950 (kpm/min)	τ_1 (sec) 650—1 300 (kpm/min)	T_D (sec) 0—650 (kpm/min)	T_D (sec) 300—950 (kpm/min)	T_D (sec) 650—1 300 (kpm/min)	L×10³ 0—650 (kpm/min)	L×10³ 300—950 (kpm/min)	L×10³ 650—1 300 (kpm/min)
KK	95.8 ± 8.7	43.8 ± 3.0	82.6 ± 4.2	—9.1	9.7	8.4	20.9	16.8	5.9
LM	96.5 ± 13.3	63.9 ± 7.4	128.0 ± 10.0	3.1	10.4	3.8	37.4	40.9	7.1
BN	74.0 ± 4.9	97.1 ± 6.3	96.0 ± 6.9	6.2	—1.6	3.8	12.6	9.0	11.1
JN	92.2 ± 8.0	77.1 ± 5.5	88.0 ± 6.4	20.9	—1.8	0.1	14.4	15.8	14.0
LP	50.8 ± 3.9	82.7 ± 7.3	88.7 ± 8.6	16.9	14.0	3.1	20.3	18.3	21.4
KS	175.4 ± 27.1	35.6 ± 3.0	64.5 ± 3.8	—34.9	20.3	15.8	24.1	23.9	10.7
Mean	97.5	66.7	91.3	0.5	8.5	5.8			
SE	17.2	9.6	8.5	8.3	3.6	2.3			
Weighted* ensemble means	96.4 ± 4.2	67.0 ± 2.3	101.2 ± 3.5	2.5	7.0	1.6	3.9	3.3	1.9

* Best fit dynamic parameters obtained after analog summing of weighted individual responses

transient responses than for the individual transient responses, demonstrates the noise reducing effect of the weighted summing procedure, *i.e.*, the suppression of random fluctuations

By application of the Student *t*-test to intra pair mean differences it was shown that for all step changes in work load the τ_1-values were significantly larger for ventilation than for heart rate ($p < 0.05$) except for the negative step 1300→650

TABLE V First order best fit parameters for ventilatory responses to negative steps in work load τ_1 = time constant T_D = time delay, L = value of associated loss function

Subject	τ_1 (sec) 650—0 (kpm/min)	τ_1 (sec) 950—300 (kpm/min)	τ_1 (sec) 1 300—650 (kpm/min)	T_D (sec) 650—0 (kpm/min)	T_D (sec) 950—300 (kpm/min)	T_D (sec) 1 300—650 (kpm/min)	L×10³ 650—0 (kpm/min)	L×10³ 950—300 (kpm/min)	L×10³ 1300—650 (kpm/min)
KK	61.4 ± 7.2	49.2 ± 4.1	64.2 ± 4.9	5.2	9.0	22.1	46.1	24.2	18.4
LM	53.5 ± 4.7	26.8 ± 4.6	24.1 ± 3.1	26.6	39.4	42.6	26.8	66.4	48.7
BN	108.8 ± 8.0	111.6 ± 13.8	84.1 ± 6.2	26.1	17.2	—34.3	8.6	29.9	17.2
JN	114.1 ± 11.0	63.1 ± 7.6	47.6 ± 3.3	7.9	13.2	—7.9	15.8	46.0	17.8
LP	48.2 ± 3.3	84.3 ± 4.9	67.2 ± 4.7	22.6	—2.9	14.9	16.2	8.6	14.2
KS	99.5 ± 7.1	77.9 ± 7.5	72.8 ± 5.2	2.7	—1.1	1.1	9.5	25.6	15.2
Mean	80.9	68.8	60.0	15.2	12.5	6.4			
SE	12.1	12.0	8.7	4.5	6.3	10.8			
Weighted* ensemble means	88.4 ± 3.7	67.3 ± 3.0	73.4 ± 2.9	10.1	2.5	0.4	3.9	5.6	4.4

* Best fit dynamic parameters obtained after analog summing of weighted individual responses

TABLE VI First order best fit parameters for heart rate responses to positive steps in work load τ_1 = time constant, T_D = time delay, L = value of associated loss function

Subject	τ_1 (sec)			T_D (sec)			$L \times 10^3$		
	0—650 (kpm/min)	300—950 (kpm/min)	650—1 300 (kpm/min)	0—650 (kpm/min)	300—950 (kpm/min)	650—1 300 (kpm/min)	0—650 (kpm/min)	300—950 (kpm/min)	650—1 300 (kpm/min)
KK	9.8±0.5	19.2±0.5	47.0±1.9	4.4	1.5	—6.7	4.2	1.3	4.1
LM	15.8±0.9	30.8±1.3	34.6±1.0	0.1	—4.8	—1.4	6.3	4.7	2.3
BN	13.8±0.6	15.3±0.7	36.4±1.1	2.6	2.2	—3.3	3.4	4.0	2.5
JN	10.2±1.2	19.4±1.0	33.7±1.7	6.2	—1.8	—4.2	20.8	6.2	7.2
LP	13.6±0.7	18.3±1.3	31.5±1.2	2.7	5.9	2.2	4.5	10.5	4.2
KS	9.1±0.7	27.8±0.9	30.8±0.8	1.6	—6.9	—4.3	8.3	3.2	1.8
Mean	12.1	21.8	35.7	2.9	—0.7	—3.0			
SE	1.1	2.5	2.4	0.9	1.9	1.2			
Weighted* ensemble means	10.9±0.3	20.9±0.6	35.7±0.9	3.9	0.3	—3.3	1.3	1.7	1.6

* Best fit dynamic parameters obtained after analog summing of weighted individual responses

kpm/min where the difference was not statistically significant. Whereas τ_1 for the heart rate responses increased with the work level from which positive step changes in work load were initiated, no such tendency was observed for the ventilation. Thus with equal positive steps in work load initiated from 0, 300, and 650 kpm/min τ_1 was 11, 21, and 36 sec for heart rate and 96, 67, and 101 sec for ventilation when computed on the mean transient responses. With negative steps of equal magnitude

TABLE VII First-order best fit parameters for heart rate responses to negative steps in work load τ_1 = time constant, T_D = time delay, L = value of associated loss function

Subject	τ_1 (sec)			T_D (sec)			$L \times 10^3$		
	650—0 (kpm/min)	950—300 (kpm/min)	1 300—650 (kpm/min)	650—0 (kpm/min)	950—300 (kpm/min)	1300—650 (kpm/min)	650—0 (kpm/min)	950—300 (kpm/min)	1300—650 (kpm/min)
KK	27.4±1.4	31.3±1.1	41.0±0.9	2.4	0.1	5.2	7.3	3.1	1.4
LM	59.5±4.0	39.1±2.3	80.3±2.9	—23.0	—2.4	2.7	11.5	9.4	1.7
BN	38.2±2.1	25.6±1.5	21.4±0.6	1.8	1.8	2.0	8.4	8.8	1.8
N	45.2±3.6	19.8±0.9	43.1±1.4	—10.5	2.6	3.1	17.4	4.6	2.8
LP	34.1±2.2	40.7±1.6	35.1±1.3	—9.1	—8.8	1.5	12.1	4.5	3.5
KS	28.6±2.2	32.9±2.1	36.5±1.5	—1.8	0.3	—1.2	15.2	11.8	4.7
Mean	38.8	31.6	42.9	—6.7	—1.1	2.2			
SE	4.9	3.2	8.1	3.9	1.7	0.9			
Weighted* ensemble means	27.9±0.9	28.8±0.6	40.4±0.5	—0.8	0.1	0.9	2.2	1.2	0.4

* Best fit dynamic parameters obtained after analog summing of weighted individual responses

initiated from 650, 950, and 1300 kpm/min, τ_1 was 28, 29, and 40 sec for heart rate and 80 67, and 73 sec for ventilation The increase in τ_1 for heart rate with increasing initial work level was statistically significant ($p<0.05$) for the positive step responses Linear regression analysis showed that for the individual the τ_1 values for ventilation tended to become longer the shorter the τ_1 values for the heart rate, although this inverse relationship was statistically significant only for the negative step 1300→650 kpm/min

Inspection of the individual τ_1 values for heart rate in Table VI and VII shows that for any of the three steps (positive or negative) in work load τ_1 was considerably longer for the off transients than for the on transients, and also that this difference became smaller the higher the work intensity Thus, the mean difference was 27 sec for the steps between 0 and 650 kpm/min ($p<0.01$), 10 sec for the steps between 300 and 950 kpm/min ($p<0.05$) and 7 sec for the steps between 650 and 1300 kpm/min No such consistent differences in τ_1 between on and off transients were observed for ventilation (Table IV and V)

Second-order time constants When the second-order model was fitted to the mean transient responses for ventilation application of Cholesky s orthogonality test (see Householder 1949) showed that the order of the function was too high For heart rate on the other hand the second-order function yielded a better fit than the first order function since it could be shown by a statistical F test that the L values became significantly reduced ($p<0.01$) for the responses to all positive and negative steps except for the negative step 650→0 kpm/min

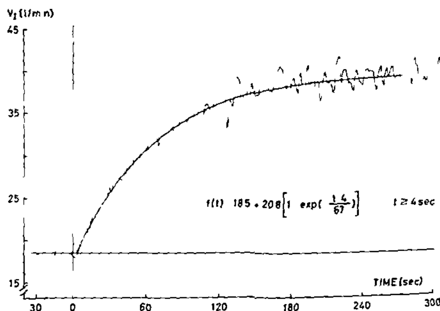


Fig 3 Mean transient response of ventilation (6 subjects) to a positive step change in work load (300→950 kpm/min) with corresponding best fit first order exponential function shown as computed for the first 270 sec

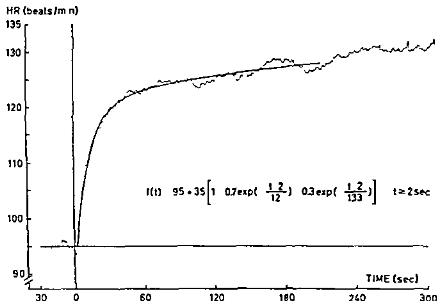


Fig 4 Mean transient response of heart rate (6 subjects) for the same step change in work load as in Fig 3 Best fit second-order exponential function shown for first 210 sec

Fig 3 shows the first order best fit function for the mean transient response of ventilation to the positive work load step 300→950 kpm/min In Fig 4 is shown the second order best fit function for the corresponding response of heart rate The curve fittings for ventilation and heart rate were limited to the first 270 and 210 sec respectively as explained on p 57

The time constants (τ_1 and τ_2) relative amplitudes (a_1 and a_2) time delays (T_D) and associated minimal L-values for the second order best fits to the heart rate responses are given in Table VIII When the initial work level was increased from 0 kpm/min to 300 and 650 kpm/min the time constant of the fast component (τ_1) of the positive step responses remained essentially unchanged (range 9 0—11 7 sec) while that of the slow component (τ_2) varied between 1 8 and 3 7 min The share of the slow component (a_2) in the total response increased and amounted to about 19 30 and 47 % respectively With the negative step changes in work load a_2 likewise increased and amounted to 0 50 and 93 % when the initial work level was 650 950 and 1300 kpm/min respectively The corresponding values for τ_1 were approximately 28 12 and 3 sec An interesting feature is that the time constants of the slow component (τ_2) were considerably longer for the positive than for the negative step responses (Table VIII)

Time delays The estimates of pure time delays obtained with the exponential identification procedure are given in Tables IV—VIII From the values given in these tables the pure time delays inherent in the recordings of ventilation and heart rate here assumed to approximate 3 0 and 0 5 sec respectively should be subtracted

TABLE VIII Second order best fit values of time constants (τ_1 and τ_2), relative amplitudes (a_1 and a_2), and time delays (T_D) for mean transient responses in heart rate with associated minimal values of loss function (L)

Step change in work load (kpm/min)	τ_1 (sec)	a_1 (%)	τ_2 (sec)	a_2 (= 100 − a_1) (%)	T_D (sec)	$L \times 10^{3}$
0—650	9.0 ± 0.5	81.4	219.1 ± 193.8	18.6 ± 8.0	4	0.95
300—950	11.7 ± 0.6	70.5	132.9 ± 34.6	29.5 ± 1.6	2	0.50
650—1 300	10.6 ± 0.3	53.5	110.9 ± 6.4	46.5 ± 0.5	3	0.10
650—0	27.9 ± 0.9	100.0		0.0	−1	2.21
950—300	12.0 ± 1.7	49.9	51.9 ± 6.5	50.1 ± 6.3	2	0.76
1 300—650	3.2 ± 1.4	7.5	41.8 ± 0.8	92.5 ± 1.1	2	0.36

It should be noted that since the T_D-estimates were derived by fitting mathematical models to the experimental data, they merely represent, from a mathematical point of view, adjustment parameters for obtaining the best fit. Especially in cases where the order of the selected exponential function is too low, these estimates may therefore differ from the true time delays that can be measured by close inspection of recordings showing the initial phases of the "on"- and "off"-transients (see further 'Discussion' p. 69).

Transitions between rest and 0 kpm/min

For comparison, running mean responses in ventilation and heart rate at the transition from rest to loadless pedaling at the beginning and at the transition in the opposite direction at the end of the experimental protocol were also studied. Close inspection of the tracings showed that at the onset of loadless pedaling both ventilation and heart rate increased rapidly and without any time delays. Whereas the heart rate showed a 50 per cent overshoot (*cf.* Fig. 5 and 6) before approaching a new level about 16 beats/min above the resting level, no such overshoot was observed for the ventilation (Fig. 6). At the cessation of pedaling both ventilation and heart rate showed an immediate rapid fall before gradually approaching their final levels.

Discussion

A primary objective of this investigation was to find out to what extent the dynamics of the ventilation and heart rate responses to step changes in work load are dependent on the initial level of work intensity. The experimental program was therefore designed to include positive and negative step changes of the same magnitude (650 kpm/min) initiated from different work levels, *viz.* positive steps from 0, 300, and 650 kpm/min, and negative steps from 650, 950, and 1300 kpm/min. Since even the highest work intensity was well below the subject's predicted maximal aerobic work

capacity the present results and conclusions refer only to submaximal work with normal or only slightly raised lactate levels

The analysis concerned intact normally operating systems exposed to muscular exercise stress with variations in work intensity sufficient to cause considerable deviations of the variables under study although still within the approximately linear region of operation A new method for exponential identification of dynamic system parameters was used which is suitable for computer execution and operates efficiently even in the presence of noise and spontaneous fluctuations superimposed on the basic response signals

Blood lactates and rectal temperature were not measured However these variables did not show significant increases in a previous series of experiments (Wigertz 1970) in which the present subjects participated and where the average work load over similar periods of time as in the present experiments was somewhat higher It can therefore be assumed that these factors were not critical for the dynamics observed for the changes in ventilation and heart rate in the present experiments Moreover heart rate showed only a small drift over the duration of the experiment (about 71 min) the difference between the mean stable state values for loadless pedaling obtained at the beginning and end of the experimental protocol on the average corresponding to less than one beat/min for each 6 min work period

The investigation also included transitions from rest to zero-load pedaling and from zero-load pedaling to rest (at the beginning and end of the experimental protocol) The responses to these transitions will be discussed separately (p 69) since the step in work load was smaller than the standard step of 650 kpm/min used in the main experiments Nor were they analyzed mathematically since they showed a very fast initial change which did not lend itself to mathematical analysis

General features of transient dynamics of ventilation and heart rate with step changes from one work load to another

The time constants and time delays presented in Tables IV—VIII for the ventilatory and heart rate responses to step changes in work load were obtained by determining the dynamic parameters for the first and second-order functions that satisfied the criterion for the best mathematical fit to the experimental data *i e* minimization of the L-value (see above Identification of dynamic parameters p 57)

The finding that irrespective of the initial work level the transient dynamics of the ventilatory responses to both the positive and negative steps in work load could be accurately described by first-order exponential models whereas the heart rate responses required second order models relied on a fitting procedure that encompassed only the first 270 and 210 sec of the ventilatory and heart rate responses respectively (*cf* p 57 and Fig 3 and 4) It was therefore of interest to determine how accurately the further time courses of the two variables could be predicted For this purpose the predicted time averages over the 6th (5th) minute of each step response in ventilation and heart rate (stable state responses) were compared with

TABLE IX. Differences between predicted and measured responses of ventilation ($\Delta\dot{V}$) and heart rate (ΔHR) derived from time averages over the 6th minute following positive and negative step changes in work load

Step change in work load kpm/min	$\Delta\dot{V}$ %	$\Delta\dot{V}$ l/min	ΔHR %	ΔHR beats/min
0—650	−1.1	−0.2	−8.9	−3.1
300—950	0.0	0.0	−5.7	−2.1
650—1 300*	+0.4	+0.1	−2.9	−1.2
650—0	−2.1	−0.4	−12.1	−4.2
950—300	−2.3	−0.5	+1.5	+0.5
1 300—650	−4.1	−1.3	−0.8	−0.3

* Values refer to the 5th min.

the corresponding means of the experimental response data. The differences between predicted and measured data, expressed in absolute values and as percentages of the final or stable state responses, are given in Table IX. From the good agreement between predicted and measured data it may be inferred that the models arrived at for the two variables are representative also for their time courses beyond the first 270 and 210 sec.

The observation that the corrected (*cf.* p. 63) time delay estimates for mean transient responses ranged between −2.6 and 7.1 sec for ventilation and between −1.5 and 3.5 sec for heart rate suggests that pure time delays can be considered relatively unimportant for the description of the adjustments of these variables to step changes in work load. This confirms the notion that both the ventilatory and heart rate responses start well before blood-borne humoral agents from working muscles have time to act on arterial or medullary chemoreceptors.

Analysis of the transient dynamics of the ventilatory and heart rate responses to step changes in work load can be expected to yield important information about the *dynamic properties of underlying systems*. Other types of work load input forcings which may be utilized for the same purpose are sinusoidal and ramp inputs. Results from such investigations with sinusoidal inputs have been reported previously (Wigertz 1968, 1970). As long as the systems studied are reasonably linear as assumed in the present analysis, one would expect similar dynamics in their responses to the above-mentioned input wave forms. In fact, sinusoidal inputs yielded qualitatively equivalent results, in that best fits were obtained with first order functions for ventilation and second order functions for heart rate, and with time constant estimates that were of the same order of magnitude as obtained in the present study with *step responses between 300 and 950 kpm/min*. Likewise, pure time delays were found to be negligible.

Cardus and Zeigler (1968) have recently described a mathematical nonexponential model of the heart rate response curves obtained at the start and end of constant load exercise, which was claimed to fit the recovery data better than an exponential model. Heart rate was measured as averages over consecutive 15 sec intervals, how-

ever, which would tend to distort the actual time courses Moreover, no attempt seems to have been made to fit a second order exponential model to the experimental data Contrary to the present observations, Galletti, Haab and Fleisch (1956) and Aepli, Pitteloud and Fleisch (1959) found that the transients in ventilation at start and end of exercise may be represented by the sum of two exponential functions of time The discrepancy in results may be explained in part, as due to the fact that the respiratory minute volume was measured breath by breath in the present investigation but only at one minute intervals in the study of Aepli *et al* Teillac and Lefrançois (1962), on the other hand, found that the ventilatory recovery is described by a simple exponential curve after mild exercise, and by a two-exponential curve after more intense exercise

It is now generally held that the changes of cardiac output in the steady state of supine leg exercise occur primarily through adjustments in heart rate, the stroke volume remaining relatively constant except at heavy or exhaustive work (Rushmer 1959 Bevegård Holmgren and Jonsson 1960, 1963, Wade and Bishop 1962) Jones *et al* (1970) have recently shown that this is the case also during the transient phases of supine exercise *In the present study, with the subjects exercising in the supine* position, the time courses of the changes in cardiac output should therefore be similar to those in heart rate, and can thus be assumed to show the dynamic characteristics of a second-order exponential model It is of interest to note in this connection that, for subjects exercising in the upright position, Cerretelli Sikand and Farhi (1966) report data that identify two components in the cardiac output response following the onset of exercise, but only one component during recovery

Influence of initial work level on transient dynamics

With rest as the control condition Cerretelli *et al* (1966) found the time constants for the changes in both ventilation and cardiac output to be independent of the exercise level Jones *et al* (1970), on the other hand, report that the rate of increase

TABLE X Predicted changes in ventilation and heart rate expressed as percentages of final (stable state) responses attained 0 5, 1 0 and 2 0 minutes after the onset of positive and negative step changes in work load from different work levels *

Step change in work load (kpm/min)	Ventilation			Heart rate		
	0 5 min	1 0 min	2 0 min	0 5 min	1 0 min	2 0 min
0—650	25	45	70	79	85	89
300—950	29	55	82	70	80	88
650—1 300	25	44	69	59	72	84
650—0	20	43	71	67	89	99
950—300	34	57	83	66	83	95
1 300—650	33	56	80	53	77	95

* Values derived from mathematical analysis of mean transient responses

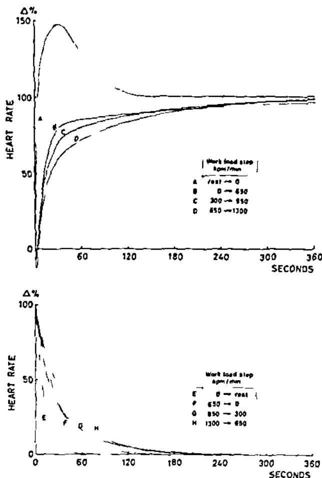


Fig 5 Predicted heart rate responses to positive and negative step changes in work load with second order best fit parameters (excepting the curves showing transitions between rest and 0 kpm/min, see text p 64

in heart rate and cardiac output becomes slower the larger the step increase in work load According to Aepli *et al* (1959) the speed of ventilation adjustments during onset of exercise and recovery is independent of the work intensity, and slower during exercise than in recovery In the present study it was observed that with the same step change in work load, the time constant of the ventilatory response was independent of the initial work level However, in early exercise and recovery the readjustment in heart rate was slower the higher the initial work level (Fig 5) Table X shows the changes both in ventilation and heart rate, expressed as percentages of their final or stable state responses, 0 5 1 0, and 2 0 min following step changes in work load initiated from different work levels The values given in the table were derived from the dynamic parameters of the mathematical model that yielded the best fit to the mean transient data It can be seen that in early exercise and recovery the readjustment in heart rate was considerably faster than that in ventilation, irrespective of the initial exercise level

The values for time constants and relative amplitudes of the heart-rate responses

(Table VIII) indicate that elevation of the initial work level was clearly associated with an increase in the relative share of the slow component This observation suggests that the share of the slow component becomes larger the closer the heart rate approaches its upper limit Such a change of the relationship between the fast and slow components might also explain the observation of Jones *et al* (1970) that the responses in heart rate and cardiac output become slower the larger the increase in work load.

Distinction between rest and zero load pedaling

Most investigators have observed an immediate abrupt increase in ventilation at the start of exercise (Krogh and Lindhard 1913 Dejours 1959 for reviews see Matell 1963 Dejours 1967) whereas others have found a gradual and sometimes delayed response (Craig Cummings and Blevins 1963 Beaver and Wasserman 1968) In the present study with step changes from one work load to another and the subjects already pedaling immediate abrupt increases or decreases in ventilation were evident only in rare instances corresponding to those with markedly negative T_D values (*cf* Table IV and V) Usually the early ventilatory adjustments were gradual In contrast abrupt changes in ventilation regularly occurred with the transitions from rest to loadless pedaling at the beginning (Fig 6) and from zero load pedaling to rest at the end of the experimental protocol Thus the demonstration of the so-called fast (neural) component in dynamic exercise hyperpnea (*cf* Dejours 1964) may require that motionless resting serve as the control condition

Beaver and Wasserman (1968) found that the extra oxygen consumed during loadless pedaling at 60 rpm in the seated position was equivalent to a work rate of 275 kpm/min Calculated from data presented by Whipp and Wasserman (1969) a value of about 140 kpm/min is obtained In the present experiments the average stable state values for ventilation and heart rate during loadless pedaling were 4 6 l/min and 16 beats/min higher than during rest These values correspond to 120 and 270 kpm/min if it is assumed that the regression lines (p 57) are valid also in the range where the work output cannot be measured Roughly estimated loadless pedaling at 60 rpm would then be equivalent to a work rate of some 200 kpm/min Thus when using motionless resting as the base line or control condition mild dynamic exercise may be sufficient to make the fast ventilatory component stand out

Ever since the observations communicated by Krogh and Lindhard in 1913 it is well recognized that the start of dynamic exercise is accompanied by an immediate fast rise in the heart rate In the present experiments the most abrupt changes in heart rate were seen—as was the case for ventilation—when loadless pedaling started or ended with motionless resting as the control condition (Fig 5 and 6) It may be of interest to note that the observation that both ventilation and heart rate increase more abruptly when exercise is initiated from rest than from no load pedaling was made already in 1928 by Paterson In the present experiments loadless pedaling was always started and stopped on verbal command Hence it cannot be settled whether the abrupt responses of ventilation and heart rate seen at the onset and

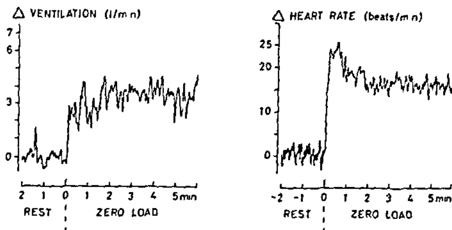


Fig 6 Mean transient responses in ventilation and heart rate at the transition from rest t loadless pedaling at the beginning of the experimental protocol

cessation of such pedaling were manifestations of conditioned reflexes associated wit the countdown or of other neurogenic influence of cerebral and/or periphera mechanoreceptive origin

With the positive steps in work load initiated from different load levels and wit the subjects already pedaling the rise in heart rate followed a pattern that was in th main similar to that described by many other investigators who have used motion less resting as the control condition (cf Harris and Porter 1958 Davies and Harri 1964) However close inspection of the analog heart rate tracings magnified by th aid of the X Y recorder revealed that the rise was usually preceded by an initia short lasting (2—4 sec) drop which tended to become less marked the higher th initial work load (Fig 5) The cause of this initial drop in heart rate which did no occur with transition from rest to loadless pedaling requires further study

Dissociated behavior of ventilatory and circulatory responses

The more important differences that were observed with regard to the transient dynamics of ventilation and heart rate with step function work load inputs can be summarized as follows *1)* Whereas the transient responses of ventilation could be accurately described by first order models those of heart rate required second-order models *2)* The ventilatory responses were slower than those of the heart rate both with positive and negative step changes in work load and irrespective of the initial work level *3)* With the step changes initiated at different levels of stable state exer cise the share of the slow component in the total heart rate response increased with initial work level whereas th latter had no consistent influence on the rapidity of the ventilatory response *4)* Individual responses of ventilation showed a tendency to become slower, the faster the responses of heart rate

For reasons outlined above (p 67) the dynamics of the cardiac output responses may be assumed to have been similar to those of the heart rate responses If this

assumption is correct, it is evident that the underlying respiratory and circulatory control systems, although presumably coupled in order to account for the ultimate control of gas exchange, exhibit a dissociation with regard to their dynamic properties This is in agreement with conclusions previously drawn from results obtained with sinusoidal work load inputs (Wigertz 1970)

Appendix

Identification of time constants of a linear system from step responses

Consider an approximately linear system with likewise approximately constant (stationary) dynamic characteristics Let the input be a step applied to the system at time $t = 0$ The output, denoted y_i, is observed at times $t = t_i$, $i = 1, 2, \ldots, N$

With this model for the system the following function is adopted to be fitted to observed data

$$f(t, \theta) = a_0 + \sum_{k=1}^{n} a_k \exp(-t/\tau_k) \tag{1}$$

where $\theta = (a_0, a_1, \ldots, a_n, \tau_1, \ldots, \tau_n)$,
a_0 is the stable state value of the function for large t, and $\tau_1 > \tau_2 > \ldots > \tau_n > 0$

Errors of the observations are assumed to be independent and random with zero means and equal, finite variances

This implies that observed data in average are identical with function values i e

$$E(y_i) = f(t_i, \theta) \text{ for all } i \tag{2}$$

The fitting procedure is carried out with aid of digital computers in two steps comprising the derivative method and an iterative least-squares method

Derivative method

For large time ($t > 3\tau_2$)

$$f(t) \simeq a_0 + a_1 \exp(-t/\tau_1) \tag{3}$$

and the first derivative

$$\frac{df(t)}{dt} \simeq -\frac{1}{\tau_1} a_1 \exp(-t/\tau_1) \tag{4}$$

From (3) and (4) we get

$$f(t) \simeq a_0 + \tau_1 \left[-\frac{df(t)}{dt}\right] \quad (t > 3\tau_2) \tag{5}$$

Exchange the values of f(t) for $t = t_i$ with the observed values y_i $i = 1, 2, \ldots, N$ Calculate $-y_i = -\frac{\Delta y_i}{\Delta t}$ for $i = 2, 3, \ldots, N-1$

Estimates of the parameters a_0 and τ_1 ($\tilde{a}_0$, $\tilde{\tau}_1$) can then be obtained by means of linear regression analysis of the variables y and $-y$ and are simply the coefficients of the regression equation

$$y_i \simeq \tilde{a}_0 + \tilde{\tau}_1(-y_i) \quad i = b, b+1, \ldots, N-1, \tag{6}$$

where b denotes the lower time boundary $t = t_b$ for which the maximal value of the correlation coefficient between y and $-y$ is obtained Then using the estimate of τ_1

$$x(t_i) = \exp(-t_i/\tilde{\tau}_1) \quad i = b, b+1, \ldots, N-1 \tag{7}$$

is calculated and for large t values ($i > b$), substituting (7) into (3) we obtain

$$f(t_i) \simeq a_0 + a_1 \cdot x(t_i) \tag{8}$$

or

$$y_i \simeq \tilde{a}_0 + \tilde{a}_1 \cdot x(t_i) \quad i = b, b+1, \ldots, N-1 \tag{9}$$

The estimates of a_0 and a_1 are calculated as y intersection and slope respectively of the line for y_i and $x(t_i)$ for $i = b, b+1, \ldots, N-1$

A new set of values is formed by subtraction of the exponential component just estimated from all observed data values (peeling off)

$$y_{1i} = y_i - \bar{a}_1 \exp(-t_i/\bar{\tau}_1) \quad i = 1, 2, \quad , b \tag{10}$$

From this new set of data the parameters for the second exponential component (a_2 τ_2) can be estimated by repeating the same procedure The peeling off should be carried on until a set of residual data values y_{ai} is obtained, from which no more exponentials can be separated

The row vector of estimates ($\bar{\theta}$) is not a least-squares estimate, because both variables y and —y in the linear regression analysis are subject to errors and these errors are not uncorrelated

The iterative least squares method is then used, with the derivative method estimates $\bar{\theta}$ as starting values, in order to improve the estimates The model function (1) is approximated by means of truncated Taylor series where the parameters are represented as sums of the starting values and [illegible]

[illegible] and the procedure repeated iteratively until the correction vector becomes smaller than a limit vector chosen so small that the properties of the parameter estimates ($\hat{\theta}$) approach those of least squares estimates

Variance of parameter estimates

Statistical properties and accuracy of the estimates can be evaluated by analyzing the residuals and calculating the covariance matrix

The residuals i e the series of differences

$$r_i = y_i - f(t_i, \theta) \quad i = 1, 2, \quad , N \tag{11}$$

are analyzed by checking the histogram and by X^2 test A corresponding loss function

$$V = 1/2 \sum_{i=1}^{N} r_i^2 \tag{12}$$

is calculated This combined indicates how well the adopted model function fits the observed step response data

[illegible]

for [illegible]

$$L = \frac{2\ V}{N-2n-1} \left[\sum_{k=1}^{n} a_k\right]^{-2} \tag{13}$$

For the first order model (n = 1 the number of parameter estimates 2n+1 = 3) (13) becomes

$$L = \frac{2\ V}{N-3} \quad \frac{1}{a_1^2} \tag{14}$$

The variances of the estimates are obtained from the main diagonal elements of the covariance matrix set up in accordance with the least squares principle

Time delay

The time delay parameter (T_D) which enters experimental data as a time shift is calculated after the fitting procedures have been carried out The methods for estimating T_D are different for first and second order fittings

First order Time delay is calculated according to the formula

$$T_D = T_{D0} + \tau_1 (\ln |a_1| - \ln |a_z - a_0|) \tag{15}$$

where T_{D0} is a starting value of time delay chosen after visual inspection of recorded curves and a_z is the mean amplitude from which the step response is started The amplitude estimate is corrected according to the formula

$$a_{1\ corr} = a_z - a_0 \tag{16}$$

Second order T_D cannot be calculated explicitly in this case but is found as the time shift for experimental data giving least squares estimates of desired parameters according to the criterion

$$|a_2 - a_0 - a_1 - a_3| = \text{Min} \tag{17}$$

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Uptake of Serotonin in Blood Platelets in vitro. I: The Effects of Chloride

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O LINGJÆRDE JR

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Abstract

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location of the carrier from the inner to the outer surface of the membrane

Blood platelets can absorb relatively large amounts of serotonin (5 hydroxytryptamine, 5HT) from the surrounding medium Two different mechanisms are thought to be involved One so-called active" mechanism following a Michaelis Menten type kinetics and one passive' mechanism, having the characteristics of simple diffusion (Born and Gillson 1959 Hughes and Brodie 1959 Crawford 1967) Since the passive uptake is small at low (and probably physiological) substrate concentrations the "active' uptake is likely to be the physiologically more important

Practically all 5HT in the platelets is stored in special dense osmiophilic granules (Tranzer *et al* 1966 Da Prada *et al* 1967 Bak *et al* 1967) The uptake therefore takes place in 2 steps (1) uptake through the plasma membrane and (2) uptake in the storage granules Even at relatively high total uptake rates the exogenous 5HT is rapidly distributed in a way similar to the endogenous 5HT *i e* stored in the granules (Tranzer *et al* 1966 Ahtee *et al* 1968, Solatunturi and Tuomisto 1968)

The rate-limiting step in the over-all uptake process at low substrate levels is therefore likely to be the passage through the plasma membrane

In a previous, preliminary communication the author reported that the 5HT uptake in human blood platelets in vitro is dependent on the presence of chloride in the incubation medium (Lingjærde 1969 a) Chloride could be partly replaced by other relatively small, unphysiological anions such as bromide or nitrite In the present communication, the role of chloride in the active uptake process will be further elucidated, and an uptake model which is in agreement with the experimental data obtained will be discussed The effects of other anions will be dealt with in a subsequent paper

Materials and methods

Reagents Labelled 5HT was obtained as 5 hydroxytryptamine 3 ^{14}C creatinine sulphate from The Radiochemical Center Amersham and unlabelled 5HT also as the creatinine sulphate from F Hoffmann La Roche & Co Basel The specific activity of ^{14}C 5HT was adjusted to 10 μC/μmole by adding unlabelled 5HT The ^{14}C 5HT was dissolved in 1 mM HCl and diluted so that the proper amounts of ^{14}C 5HT were always added in a volume of 20 μl to the test samples Since the incubation volume was always 2 ml prior to adding ^{14}C-5HT a correction for the added volume of ^{14}C 5HT was considered unnecessary The stability of the ^{14}C-5HT solutions were checked regularly by paper chromatography according to Pletscher *et al* (1966) Other chemicals used were of *p a* purity

Equipment Blood samples were drawn with disposable stainless steel needles 1 2 mm in [illegible] ...pellant [illegible]

meter (Model 3003) Radioscanning after chromatography was done using a Packard radiochromatogram scanner (Model 7200)

Methods Blood samples were drawn from a cubital vein of healthy persons and mixed with one tenth the volume of 3 % EDTA in 0 5 % NaCl Platelet rich plasma (PRP) was obtained by centrifuging (250 × *g*) for 15 min at room temperature Aliquots of 1 ml PRP were mixed with 1 ml isotonic NaCl and centrifuged (2500 × *g*) for 15 min at 4° C The supernatant was discarded the test tubes allowed to drain on filter paper for about 5 min and the remaining supernatant carefully wiped off with filter paper The platelet pellets were then resuspended in 2 ml of 55 mM phosphate buffer pH 7 3 (if not otherwise stated) with varying concentrations of NaCl Na_2SO_4 and K_2SO_4 to a total osmolarity of 275 to 350 mM (this variation in osmolarity does not influence initial uptake rate) The sulphate anion was used to replace chloride when appropriate because it is inactive in the actual uptake process nor has it any inhibitory effect when present together with chloride (Lingjærde 1969 b) The potassium concentration was kept constant at 5 mM and the sodium concentration at about 175 mM

Incubation was performed at 37° C in a metabolic shaker If not otherwise stated the test [illegible] ...dding ^{14}C 5HT The [illegible] ...latelets were isolated [illegible] with filter paper [illegible] ...ed by freezing over night at −20° C followed by thawing and centrifuging One half ml of the supernatant was then mixed with Bray s solution (Bray 1960) for liquid scintillation counting Blank samples were prepared by adding ^{14}C 5HT to test tubes kept in ice water just prior to centrifuging and internal standards by adding ^{14}C 5HT together with distilled water prior to freezing [illegible] ...ed from differences between duplicate samples [illegible] ...n between experiments [illegible] ...rast microscopy Platelet recovery after resuspen[illegible] with the present method In these experiments [illegible] important it was not found necessary to count [illegible] Where 5HT uptake is given in absolute values [illegible]

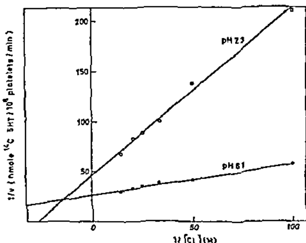


Fig 1 Double reciprocal plots of ^{14}C 5HT uptake rate versus chloride concentration at pH 6 1 and 7 3 The chloride concentration was varied in the range of 10 to 70 mM For further details on composition of medium see Methods The mixture was pre incubated for 8 min without 5HT, and thereafter incubated for 8 min with 0 4 μm ^{14}C 5HT added

Results

1 *Correlation between extracellular chloride concentration and initial uptake rate*

The dependence of uptake rate on concentration of chloride in the medium is shown in Fig 1 Since the uptake rate has been found to be highly dependent on pH, having a maximum at about pH 6 (Stacey 1961, Lingjærde 1969 a), the uptake was in this experiment also measured at pH 6 1 It is seen from the double reciprocal plots that the correlation between chloride concentration and initial uptake rate adheres to Michaelis Menten type kinetics, with apparent $K_{m\,(Cl^-)}$ 37 mM at pH 7 3 and 11 mM at pH 6 1, whereas the maximal uptake rate (V_{max}) is nearly 2 times higher at pH 6 1 than at pH 7 3

The kinetics of the chloride effect was further analysed by incubation with 4 different concentrations of 5HT at 4 different concentrations of Cl In Fig 2 a the

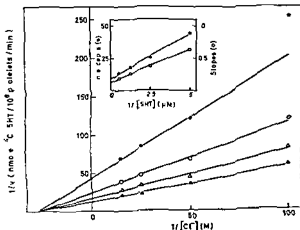


Fig 2 a. Main figure Double reciprocal plots of ^{14}C 5HT uptake versus *concentration of chloride* at different concentrations of 5HT ● 0 2 μM ○ 0 4 μM △ 0 8 μM and ▲ 2 0 μM For further details on composition of medium see Methods The mixture was preincubated for 8 min without 5HT and thereafter incubated for 8 min with ^{14}C 5HT added

Inserted figure Intercepts and slopes from the main figure plotted against 1/[5HT]

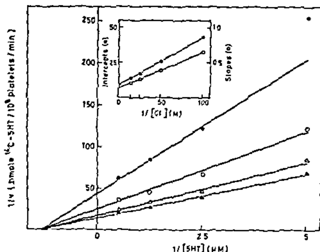


Fig 2 b Same experiment as in Fig 2 a Main figure Double reciprocal plots of ^{14}C 5HT uptake against ^{14}C 5HT concentration at different concentrations of chloride ●, 10 mM o, 20 mM, △, 40 mM, and ▲ 70 mM Inserted figure Intercepts and slopes from the main figure plotted against 1/[Cl]

double reciprocal plots of net uptakes versus Cl concentration at different levels of 5HT concentrations are shown It is seen that the apparent $K_{m\,(Cl)}$ is not influenced by the amount of 5HT Moreover double reciprocal plots of initial uptake rates versus 5HT concentration (Fig 2 b) show that the apparent $K_{m\,(5HT)}$ is independent of the amount of Cl Secondary replots of slopes and intercepts against the reciproces of the concentrations of Cl and 5HT, respectively, are shown in inserted diagrams, they all give straight lines The significance of this will be discussed below

2 The effects of preincubation with different concentrations of chloride

The decrease in net uptake of 5HT with decreasing concentrations of chloride might in part be due to unspecific changes in the platelets during incubation with unphysiologically low amounts of chloride If so there should be a decrease in uptake rate with increasing preincubation in a medium with low chloride concentration

The effects of preincubation with different chloride concentrations are shown in Fig 3 With 60 mM Cl there is no change in the uptake rate (measured for periods of 5 min) between 5 and 60 min preincubation With 10 mM Cl, there is a significant decrease in uptake rate amounting to 34 % between 5 and 60 min or 0 6 % per min on the average (The shape of the curve seems to indicate an exponential function However several independent experiments have failed to show this convincingly, because the differences in uptake rates are so small) Rather unexpectedly, preincubation with 100 mM Cl was accompanied by an increased uptake rate, the increase in this case amounting to 20 % between 5 and 60 min

The rate of decrease when preincubating with low amounts of chloride has been found to be fairly constant from one experiment to another, whereas the increase with higher concentrations of chloride has been more varying, from a few per cent to more than 30 per cent between 5 and 60 min This variation seems to be mainly due to individual differences

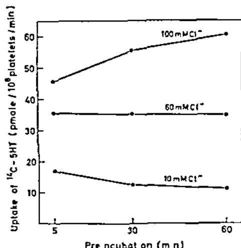


Fig 3 The effects of preincubation with different chloride concentrations on ^{14}C 5HT uptake rate After the preincubation periods shown in the figure, ^{14}C 5HT was added to a final concentration of 1 μM and the uptake allowed to proceed for 5 min For further details on composition of medium see Methods. Middle and lower graphs are from the same experiment while the upper graph is from another The absolute uptake rates are therefore not strictly comparable Each point represents the mean of two parallel samples

Although these experiments have shown that there is a definite decrease in the rate of uptake when platelets are preincubated with low chloride concentrations this decrease is not very great, and it can not explain the chloride dependency of the 5HT uptake reported above However, returning to the results presented in Fig 1 and 2, it appears that elimination of this presumably unspecific effect will call for some upward corrections of the uptake rates at lower chloride concentrations But these corrections will not be great enough to significantly alter the shape of the uptake curves, and no attempt has therefore been made to correct the values

Discussion

It has been shown that the rate of 5HT uptake in blood platelets is dependent on the concentration of chloride in the medium no net uptake taking place without the presence of chloride Since net uptake is influx minus outflux the question arises whether part of the chloride effect could be due to increased outflux in a medium with unphysiologically low concentrations of chloride The author has devised a method for measuring spontaneous outflux of ^{14}C 5HT from preloaded resuspended platelets (Lingjærde 1969 b) and with this method it has been shown that the spontaneous outflux of 5HT is not increased by lowering the chloride concentration in the medium Thus the low net uptake of 5HT in a medium with low concentration of chloride must be due to decreased influx only

Some other anions can replace chloride in the 5HT uptake process (Lingjærde 1969 a, to be dealt with in more detail in a subsequent paper) However this does not apply to anions normally present in blood in appreciable amounts such as bicarbonate or phosphate Chloride is therefore considered to be the physiological anion taking part in uptake of 5HT in platelets and the present discussion will be restricted to the effects of this anion

Before discussing the exact role of chloride in the uptake mechanism proper, there are some relevant points to be made

Firstly, 5HT in an aquous solution is present almost exclusively as a positively charged ion at physiological pH (Garattini and Valzelli 1965) To maintain ionic equilibrium within the cell, influx of 5HT must therefore be accompanied by an equivalent influx of anions, or an equivalent outflux of cations

Secondly, the ratio of intracellular to extracellular chloride in human platelets has been found to be about 0 7, and there are no indications that chloride is actively transported across the membrane (Gorstein *et al* 1967) The concentration ratio seems to agree with the assumption that chloride can diffuse freely through the platelet membrane

Thirdly, although it has been firmly established that the so called "active uptake of 5HT through the platelet membrane shows saturation kinetics, it has so far not been established whether the uptake mechanism is that of facilitated diffusion co-transport (or secondary active transport) or primary active transport (see Stein 1967 for definitions of these terms) As the commonly used term implies the uptake has usually been considered to be active' However as pointed out by Maynert and Isaac (1968) there is no firm evidence to support this view

It has recently been shown that 5HT uptake in platelets is also dependent on the presence of sodium (Lingjærde 1969 a), and the possibility is that this is a case of co-transport with sodium or chloride (or both) as the driving species The effect of sodium is not to be discussed here If chloride is passively distributed between the intra and the extra cellular space, it can not be the driving species in a co transport process because this requires an inward electrochemical gradient

Which role then does chloride play in the uptake process? Since 5HT is present as a cation, it is likely that it must be accompanied through the cell membrane by chloride to maintain ionic equilibrium However this does not explain why active uptake of 5HT does not take place in the absence of chloride (*e g* outflux of sodium or potassium ought to be equally effective) or why the effect of chloride adheres to strict saturation kinetics It seems reasonable to assume that chloride is more intimately involved in the transport process than merely to serve as an ionic equalizer'

Several hypothetical possibilities has to be taken into consideration when trying to explain the effect of chloride on 5HT transport For example there is the possibility that chloride may act as an allosteric activator of some constituent of the plasma membrane The kinetic data do not support this possibility but it can not be totally ruled out

Another possibility is that chloride has an indirect effect by influencing the membrane potential According to Nernst's law and provided that chloride can diffuse through the plasma membrane increasing extracellular concentrations of chloride will increase the membrane potential and thereby possibly stimulate the uptake of 5HT However this is not in agreement with the observed stimulatory effect of potassium (Weissbach and Redfield 1960 Rysanek *et al* 1967 Lingjærde

1969 b), which has an opposite effect on the membrane potential Furthermore, an indirect effect via the membrane potential would not be expected to give simple saturation kinetics

A third hypothesis, which seems to be the one that best suits the present data, is that the presumably carrier mediated 5HT transport can only take place if chloride is bound to, and transported by, the same carrier This hypothesis will therefore be dealt with in some detail

The carrier concept is in itself rather hypothetical, but has been found useful in explaining many transport phenomena, especially those exhibiting saturation kinetics, substrate specificity, and competitive inhibition by structurally related compounds According to these properties, the 'active' uptake of 5HT in platelets is assumed to be carrier mediated The transfer of the 5HT molecule across the membrane is then considered to proceed in 3 stages I The binding of 5HT to the carrier at the outer surface II Translocation from the outer to the inner surface of the membrane III Release at the inner surface

It has been found useful in many instances to treat carrier mediated transport in accordance with enzyme kinetics (Stein 1967) Assuming that chloride and 5HT are bound to the same carrier, and that both are released at the inner surface, this mechanism could be treated kinetically as a bisubstrate reaction According to modern enzyme kinetics (Cleland 1963, Mahler and Cordes 1966), the following conclusions can then be drawn from the data in Fig 2 (1) Since the intersection points of the plots of 1/v versus 1/[5HT] or 1/v versus 1/[Cl] are lying on or very near the x axis, the binding of 5HT and Cl to the carrier is in accordance with a random mechanism, i e the binding of each species is unaffected by prior binding of the other one (2) Since the replots of intercepts on the y axis versus 1/[5HT] and versus 1/[Cl] and of slopes versus 1/[5HT] and 1/[Cl] all make straight lines both 5HT and Cl are bound only once to the carrier

Thus, according to this hypothesis Stage I in the uptake process consists of the binding of one molecule of 5HT and one molecule of Cl to be carrier Chloride is not important at this stage because the binding of 5HT is unaffected by prior binding of Cl Therefore chloride is likely to play its role in Stage II the translocation or in Stage III the release process However if extracellular chloride does not influence the binding of 5HT at the outer surface it seems rather unlikely that chloride (extracellular or intracellular) affects the release of 5HT at the inner surface Thus, it may be suggested that the main role of extracellular chloride is on the inward translocation of the carrier substrate complex

The preincubation experiments seem to indicate that the intracellular chloride concentration is also of importance for the uptake rate because preincubation with varying chloride concentrations is likely to produce alterations in the intracellular concentration of chloride (without significantly altering the extracellular concentration because the volume of the platelets is very small compared to the incubation volume) The effects of preincubation may thus be simply explained by assuming a higher rate of re translocation for the carrier Cl complex than for the free c

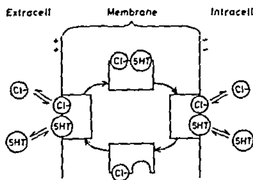


Fig 4 Simplified schematic representation of the hypothesis concerning the role of chloride in uptake of serotonin

The main features of the present hypothesis concerning the role of chloride in the 5HT uptake process are shown schematically in Fig 4 Needless to say, this hypothesis concerns only one aspect of the process and do not for example, take into account the important role of sodium (Lingjærde 1969 a)

This study was supported by a grant from Brukseier Jonn Nilsen og hustru Maja Jonn Nilsen s legat I am indebted to Dr Elling Kvamme and his coworkers for all their help during the study The skilful technical assistance of Mrs Torill Moe is gratefully acknowledged

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Cerebellar Suppression of the Autonomic Components of the Defence Reaction

By

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Abstract

Lisander, B and J Martner *Cerebellar suppression of the autonomic components of the defence reaction* Acta physiol scand 1971 *81* 84—95

Experiments were performed on anesthetized-curarized cats, where both the hypothalamic defence area and the cortex of the anterior cerebellar lobe could be stimulated while blood pressure, heart rate and muscle blood flow were recorded Cerebellar stimulation which, when performed alone, had only insignificant cardiovascular effects, considerably reduced the increases in heart rate, blood pressure and muscle blood flow elicited by standardized defence [illegible] appeared to be due [illegible]

[illegible]

differentiated interaction act in synergism from the hemodynamic point of view (see Kylstra and Lisander 1970) the cortex of the anterior cerebellar lobe tends to suppress the defence reaction by exerting an inhibitory influence on essentially all the autonomic components of this response pattern

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The hypothalamic defence reaction involving cholinergic vasodilatation in skeletal muscles and adrenergic excitation of other cardiovascular compartments, seems to interact with the baroreceptor reflexes in a differentiated way While the baroreceptor reflex inhibition of the heart is centrally more or less suppressed by the defence reaction (Hilton 1963) the reflex inhibition of systemic resistance vessels appears to be left largely unaffected This type of differentiated interaction permits a markedly enhanced cardiac output during a defence reaction, without increasing cardiac work load proportionally (Kylstra and Lisander 1970)

The cerebellum has been reported to exert a modifying influence on some autonomic reflexes besides its well known somatomotor effects Thus, cerebellectomy can enhance the pressor response to carotid occlusion and may also increase the blood pressure fall caused by carotid baroreceptor stimulation (Reis and Cuénod

1965) Further, stimulation of the cerebellar cortex can cause a blood pressure fall, provided that the baroreceptors are unloaded However, at normal levels of blood pressure and baroreceptor activity, the effects of cerebellar stimulation seem to be slight or none (Moruzzi 1940)

In the decorticate cat, sham rage may be induced by trivial stimuli and activation of cholinergic vasodilator fibres has been shown to occur at such occasions (Abrahams, Hilton and Zbrozyna 1960) The behavioural changes, as well as the concomitant rise in blood pressure and heart rate, can be inhibited by stimulation of the cerebellar cortex (Moruzzi 1947) The aim of the present study was to investigate whether, and to what extent stimulation in the cortex of the anterior cerebellar lobe may modify various components of the defence reaction as induced by topical hypothalamic stimulation Considering the above mentioned data it seems likely that several of these different autonomic components are affected, but the net effect on the muscle vascular bed is particularly interesting in this connection For example, the cholinergic vasodilatation may remain unhampered by such a cerebellar excitation but it may be diminished as well, either by a suppressed activation of the cholinergic vasodilator fibres or by a suppression of the baroreceptor inhibitory reflexes, to such an extent that a 'break-through' of the cholinergic dilatation becomes greatly impeded (Folkow, Öberg and Rubinstein 1964)

The possibility has also been considered that baroreceptor reflexes might influence the cholinergic vasodilator fibre discharge directly *A priori* this is not unlikely in the light of findings that strong carotid sinus distension can inhibit sham rage in decorticate cats (Bartorelli *et al* 1960) This suggests that baro eceptor reflexes may exert an inhibitory influence at several levels and on many functions of the central nervous system

Methods

Experiments were performed on 40 cats of both sexes After induction with ether light anesthesia was maintained by i v administration of chloralose 30—40 mg/kg b w

With the animal s head fixed in a modified Horsley Clarke stereotaxic apparatus a sharp stainless steel monopolar electrode was inserted into the hypothalamic defence area (D A) Square wave pulses were delivered by a Grass Model S5 stimulator with an intensity of 2—5 V and duration of 1—2 msec The frequency was varied between 10—120 imp/sec but was usually set at 80 imp/sec

In order to stimulate the cortex of the anterior cerebellar lobe the muscle tissue covering the occipital bone was divided in the midline and electrodes were inserted through a hole drilled in the bone The stimulations were performed either by a bipolar concentric electrode or the pulses were passed between two monopolar electrodes at a distance of 2 mm from each other overriding the midline of the vermis Due to the meandering course followed by this

Blood pressure was measured through a catheter in the femoral artery connected to a Statham P23 AC transducer writing on a Grass Polygraph Muscle blood flow was measured as the outflow from the deep femoral vein the paw circulation being excluded by a tight ligature at the ankle After passing a closed optical drop recorder device operating an ordinate writer the blood was returned to the animal *via* one of the femoral or external jugular veins In 16 experiments the leg was skinned and the calf muscles of the animal were isolated a hole was drilled in the femur and the bone marrow plugged with cotton soaked in silicone grease When

cross circulation was to be performed (8 expts), the femoral arteries and veins of the two animals were connected in such a way that the donor supplied the circulation of the isolated calf preparation and thus the sciatic nerve formed the only intact connection between the calf and the recipient. The arterial pressure of the donor was measured from a side branch of the arterial tube and venous outflow from the calf was followed by a drop recorder coupled in series with the venous tubing connecting the calf with the donor.

The vagal nerves were dissected free in the neck and placed on ligatures so that they could be cut in the course of the experiment. In 5 cats the adrenals were exposed transabdominally and ligated. In another 4 animals one of the carotid sinus regions was gently dissected free and isolated (for details see Djojosugito *et al.* 1970). After this procedure, the carotid sinus region could be exposed either to the normal pulsatile arterial pressure or to any desired steady pressure level by way of a pressure system. The contralateral sinus nerve was freed and sectioned.

In two preliminary cross circulation experiments (one of them shown in Fig. 5) the baroreceptors were stimulated by pulling on the carotids to avoid a more extensive preparation. This was carried out in a standardized manner by connecting the ligatures around the carotid arteries to a mechanic device which pulled half a centimeter at a frequency of 30 pulls per second.

The animals received gallamine iodide (Flaxedil 2—4 mg/kg) to eliminate such secondary effects on cardiovascular dynamics that could be induced by respiratory changes or other somatomotor activities in connection with the D.A. stimulations. Constant artificial respiration was maintained by means of a respiration pump. Atropine, 0.5—1 mg/kg b.w. was given to test if and to what extent, the muscle vasodilatation was of cholinergic origin. The adrenergic system was blocked by a combination of propranolol *0.5—1 mg/kg b.w.*, guanethidine *4 mg/kg* b.w. and phentolamine 1 mg/kg b.w. In the cross circulation experiments, the two latter drugs were given intraarterially in the same doses to the calf muscle preparation.

Results

I. Cerebellar influence on the defence reaction

Fig. 1 shows that the rise of blood pressure and muscle blood flow during D.A. stimulation was markedly reduced when the cerebellar cortex was simultaneously stimulated. The blood flow increase could to a major extent be blocked by atropine. In other experiments it was found that the cerebellar stimulation also reduced the heart rate increase of the defence reaction. The different parameters were suppressed

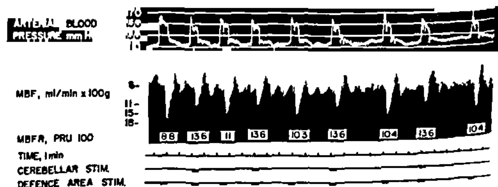


Fig. 1. Cat 3.8 kg, chloralose. Defence area (D.A.) stimulations alternating with combined D.A. cerebellar stimulations (both performed at 80 imp/sec, 1 msec and 3 V). MBF and MBFR denote muscle blood flow and muscle blood flow resistance, respectively. Note the regular reductions in vasodilatation and blood pressure rise during combined D.A. cerebellar stimulations as compared with D.A. stimulations alone.

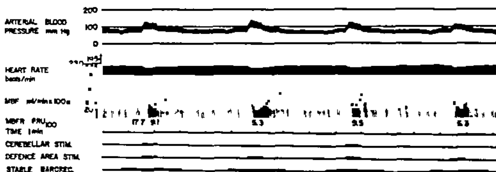


Fig 2 Cat 3 0 kg chloralose gallamine 3 mg/kg b w , artificial respiration Both vagi and [illegible] M B F and [illegible] 1 msec [illegible] to keep [illegible] hibitory effect of the cerebellar stimulation on the vasodilatation caused by the D A stimulation is still present although the only intact baroreceptor area is exposed to the same pressure during all stimulations

by the cerebellar stimulation to a varying extent from experiment to experiment, but for one and the same combination of electrode sites the effects were reproducible Fig 1 shows a representative sequence from a series of twenty identical D A stimulations where each second one was performed in combination with cerebellar stimulation The latter stimulations were always performed in or in the vicinity of the midline of the vermal cortex in the anterior lobe, close to the fissura prima If this cerebellar structure was stimulated alone it usually had no significant effect on blood pressure, heart rate or muscle blod flow, occasionally a minor fall in blood pressure was elicited

The less pronounced muscle vasodilator response to D.A activation when the cerebellum was stimulated simultaneously might be ascribed to a less pronounced increase in blood pressure and hence of baroreceptor activity with a concomitant less powerful reflex inhibition of constrictor fibre tone (see Djojosugito *et al* 1970) To test this possibility experiments were carried out in which the vagi and one of the carotid sinus nerves were cut and the pressure maintained constant in the remaining isolated carotid sinus region Fig 2 illustrates such an experiment Each D A stimulation or combined D.A cerebellar stimulation was here performed against a background of constant pressure in the carotid sinus which was so adjusted that the systemic blood pressure prior to stimulation was maintained at the resting value It is seen from Fig 2 that even with constant baroreceptor activity the muscle vasodilatation was reduced by 25—35 per cent when a cerebellar stimulation was performed concomitantly with the standardized D A stimulation

The possibility remains however that cerebellar stimulation may directly influence vasoconstrictor fibre discharge or change the stimulus response relationship

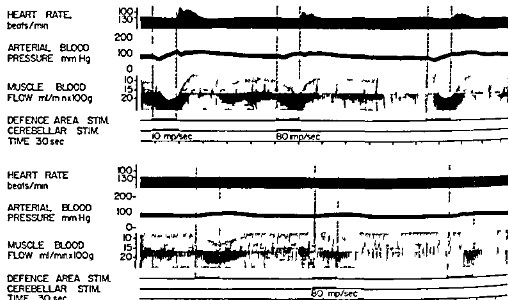


Fig 3 Cat 3 3 kg chloralose Adrenergic fibres blocked by guanethidine Effects of defence area (D A) stimulation with and without concomitant cerebellar stimulation Upper panel Before atropine administration Note the reduced muscle vasodilatation as a response to D A stimulation when the cerebellum is concomitantly stimulated at 80 imp/sec (1 msec 3 V) as compared with 10 imp/sec or when the D A is stimulated alone (80 imp/sec 1 msec, 4 V) Lower panel After administration of atropine 1 mg/kg b w Note the greatly diminished and somewhat delayed muscle vasodilatation upon D A stimulation However also now the remaining muscle vasodilatation is reduced by a concomitant cerebellar stimulation

of the baroreceptor reflex in a way that could explain the decreased muscle vasodilatation

As mentioned cerebellar stimulation alone usually did not influence resting blood pressure significantly but if sympathetic activity was raised by carotid occlusion the ensuing pressure rise could be reduced by as much as 30 mm Hg by a simultaneous cerebellar stimulation a finding which confirms the results of Moruzzi (1940)

Since it has been shown by Reis and Cuenod (1965) that the pressure drop resulting from carotid sinus distension can be enhanced by cerebellectomy attempts were made in a series of experiments (5 cats) to elucidate whether the cerebellar cortical stimulations had any influence on the reflex depressor response to a standardized baroreceptor activation No clear evidence for such a cerebellar modulation of the baroreceptor reflex mechanism was found although cerebellar stimulation regularly reduced the blood pressure by some 20 mm Hg when baroreceptor restraint on the bulbar vasomotor centres was completely abolished

It was still necessary to consider whether the decreased muscle vasodilatation produced by a concomitant D A cerebellar stimulation as compared with a D A stimulation alone, might depend upon a decreased inhibition of sympathetic tone

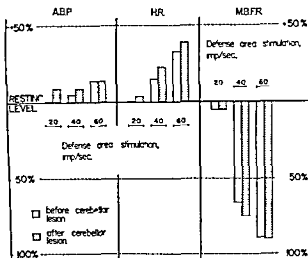


Fig 4 Cat 4 1 kg chloralose gallamine 4 mg/kg b w and artificial ventilation Effects on arterial blood pressure (A B P), heart rate (H R) and muscle blood flow resistance (M B F R) by defence area (D A) stimulation at 1 msec, 4 V and 20 40 and 60 imp/sec before and after cerebellectomy The "resting" levels (B P 110±10 mm Hg H R 180±10 beats/min and M B F R. 28±2 P R.U 100) were unaltered after cerebellectomy All changes during the D A stimulations are given as percentage deflections from the 'resting' values For further explanations see text.

in the muscles This possibility could not be entirely excluded since cerebellar stimulation in combination with D A stimulation might impose a changed stimulus response relationship of the baroreceptor reflex

To examine whether the activity in the cholinergic vasodilator fibres themselves might be reduced by the cerebellar stimulation experiments were performed where the adrenergic system had been blocked by propranolol guanethidine and phentolamine Fig 3 shows such an experiment where the vagi had been left intact although they were usually cut in these experiments At first a D A stimulation was performed together with a cerebellar stimulation (10 imp/sec) The pronounced muscle vasodilatation caused by the D.A stimulation was accompanied or slightly preceded by a minor blood pressure fall Heart rate increased somewhat in this case because of an inhibition of vagal tone Upon cessation of the D A stimulation a pronounced vagal bradycardia ensued as a rebound phenomenon If together with the D A stimulation a cerebellar stimulation of 80 imp/sec was performed the muscle vasodilatation was reduced by 40—50 per cent and it also occurred later during the D A stimulation Then a D A stimulation was performed alone and again a marked and prompt muscle vasodilatation was obtained

Then atropine was given and the effect of D A stimulation on heart rate disappeared completely However a slight and much delayed vasodilatation and blood pressure increase was still obtained Such effects could in other experiments be eliminated by ligation of the adrenals and they were probably due to a catecholamine release from these glands It is clear from Fig 3 that also these effects of D A stimulation are diminished if a cerebellar stimulation is performed concomitantly suggesting that the D A activation of the adrenal medulla can also be suppressed from cerebellar structures Also in experiments where the adrenal glands had been

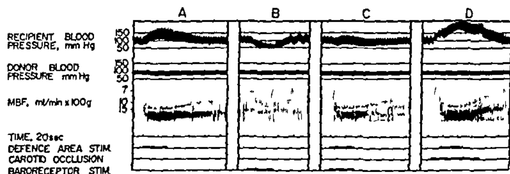


Fig 5 Cross-circulation experiment (donor 2 kg, recipient 2 4 kg, chloralose, recipient vago

1 msec, 3 5 V Note increase in muscle blood flow (M B F) at virtually constant perfusion pressure, as combined with a pronounced pressure rise in the recipient

Panel B Standardized carotid baroreceptor activation (carotid pulling) results in a blood pressure fall in the recipient but does not change muscle blood flow, indicating a complete adrenergic block in the cross-circulated limb

Panel C Simultaenous D A and baroreceptor stimulation Note reduction of blood pressure rise in the recipient as compared with panel A while the muscle blood flow increase is the same

Panel D D A stimulation during carotid occlusion Muscle blood flow increases to the same extent as in panels A and C

ligated, cerebellar stimulation suppressed the muscle vasodilatation produced by D A stimulation in animals where both α- and β-receptor blocking drugs had been given It is therefore evident that the cerebellar stimulations tend to reduce the activation of the cholinergic vasodilator fibres upon D A stimulation

A few experiments, such as that diagrammatically illustrated in Fig 4 were performed to assess whether the defence reaction could be influenced by cerebellectomy It can be seen that cerebellectomy resulted in a slight augmentation of the blood pressure rise heart rate increase and muscle vasodilatation as induced by a standardized D A stimulation, at least when this stimulation was not too intense In no experiment were the responses to D A stimulation clearly reduced as a result of cerebellectomy as compared with the situation before this procedure

II Baroreceptor influence on the cholinergic vasodilator fibre activity

Fig 5 shows an experiment where an isolated calf muscle preparation with intact autonomic innervation was cross-circulated from a donor cat, which had been given α- and β-adrenergic receptor blockers as an intraarterial infusion to the cross-circulated calf The recipient was not given any drugs but its vagal nerves were cut D A stimulation in the recipient caused a blood pressure rise and an increased muscle blood flow in the cross-circulated calf (panel A) In panel B the baroreceptors of the recipient were activated resulting in a reflex pressure fall in this

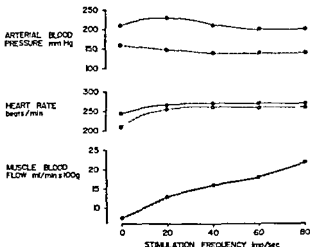


Fig 6 Cross-circulation experiment (donor 3 9 kg recipient 4 6 kg chloralose) The recipient was curarized and artificially ventilated both vagi and left carotid sinus nerve were cut while the right carotid sinus was isolated and could be excluded from the circulation and exposed to a constant pressure head The calf of the recipient was cross-circulated but with intact sciatic nerve The adrenergic vasoconstrictor fibres had been blocked by guanethidine and phentolamine given intra arterially to the cross-circulated limb

The defence area (D A) was stimulated at frequencies indicated on the abscissa and at 3 V 1 msec The frequency response curves represent the effects of these D A stimulations when all baroreceptor stations were excluded (solid lines) and when the isolated carotid sinus preparation was exposed to 250 mm Hg (hatched lines) For further explanations see text

animal However, this had no effect on the muscle blood flow in the cross circulated calf a finding confirming those reported by Folkow and Uvnas (1948) that sympathetic vasodilator fibres are not engaged in baroreceptor reflexes When a baroreceptor activation was induced in the recipient simultaneously with a D.A stimulation (panel C) the pressure increase from D A stimulation was almost annulled but the muscle vasodilatation was of the same magnitude as that in panel A Further the same D.A stimulation produced exactly the same muscle vasodilatation in the cross-circulated calf if it was performed on a background of elevated vasomotor centre activity brought about by a bilateral carotid occlusion (panel D) These findings show that the extent of activation of the cholinergic vasodilator fibres at a given D.A stimulation was not influenced by the prevailing baroreceptor activity level

It could however be argued that the D A activation may have been supramaximal so that the baroreceptors for such reasons had little chance to influence the cholinergic sympathetic vasodilatation For such reasons experiments were performed as that shown diagramatically in Fig 6 with a similar experimental setup as in Fig 5 A powerful and constant baroreceptor stimulation was here matched against D A stimulations at increasing frequencies In spite of a fairly pronounced effect on blood pressure and a very small or moderate one on a heart frequency depending on the frequency of D A stimulation (Djojosugito *et al* 1970) the intense baroreceptor activation had no influence whatsoever on the cholinergic vasodilatation In some of the experiments the impulse duration or voltage was varied instead of frequency during the D A stimulation but the results were largely the same

Discussion

The present experiments indicate that stimulation of the vermal cortex of the anterior cerebellar lobe can inhibit the defence reaction as elicited by topical hypothalamic stimulation and this inhibition affects the blood pressure rise the heart rate increase, the skeletal muscle vasodilatation and probably the adrenomedullary activation The decrease in muscle vasodilatation caused by the cerebellar stimulation is of particular interest and may, in adrenalectomized animals reflect one or both of two phenomena a reduced activation of the cholinergic sympathetic vasodilator fibres and/or an impeded reflex inhibition of vasoconstrictor fibre tone in the skeletal muscle vascular bed (see *e g* Djojosugito *et al* 1970) As to the latter possibility the reduced augmentation of blood pressure and heart activity caused by the cerebellar stimulation must imply a lower degree of baroreceptor excitation which in turn would cause a reduced reflex inhibition of vasoconstrictor fibre tone and therefore perhaps a less efficient breakthrough of the cholinergic dilatation (see *e g* Folkow Öberg and Rubinstein 1964 Djojosugito *et al* 1970)

However the decrease in skeletal muscle vasodilatation caused by a cerebellar stimulation concomitant to the D A stimulation remained almost unchanged even after stabilization of the baroreceptor activity by means of vagotomy unilateral sinus nerve section and the maintenance of a constant pressure in the remaining carotid sinus This speaks against the possibility that a reduced reflex constrictor fibre inhibition due to a decreased baroreceptor activation should be mainly responsible for the suppressed muscle vasodilatation However the possibility remains that the reflex effect of a given baroreceptor activation might be diminished by the cerebellar stimulation Moruzzi (1940) states that stimulation of the cerebellar cortex can diminish the blood pressure fall obtained by afferent vagal stimulation but in the present study it could not be shown that this was also the case with regard to the reflex fall in blood pressure caused by a standardized carotid sinus distension In order to further test if a decreased inhibition of adrenergic nervous tone could be the main mechanism by which the muscle vasodilatation was suppressed by cerebellar stimulation the adrenergic system was blocked the adrenals ligated and the vagi cut in some animals Thus under these conditions the cholinergic dilator fibres comprised the only efferent autonomic pathway of importance that could be activated by the D A stimulation However also in this situation cholinergic muscle vasodilatation was inhibited by cerebellar stimulation and consequently this stimulation must have caused a reduction of the impulse activity in the cholinergic dilator fibres as caused by the D A activation To our knowledge this is the first report on a *neurogenic inhibitory influence on sympathetic vasodilator fibre activity* The present experiments do not however exclude that the other mentioned mechanisms to some extent contribute to the reduction of the muscle vasodilatation caused by the cerebellar stimulation It should also be mentioned that it is possible that the baroreceptor reflex may be influenced by cerebellar stimulation under other experimental conditions for instance when activated by a pulsating pressure

At the frequencies for cerebellar stimulation primarily used in this study (50—100 imp/sec), a blood pressure reduction was obtained in animals whose baroreceptors had been inactivated The only corticofugal neurons of the cerebellum emanate from the Purkinje cells and these convey only inhibitory responses, cortical stimulation may however, cause inhibitory as well as excitatory responses in the cerebellar nuclei and the nucleus of Deiters The excitatory responses may be due to the fact that electrical stimulation causes concomitant antidromic activation of afferent neurons that by means of collaterals seems to reach both the cerebellar cortex and the cerebellar nuclei and/or the nucleus of Deiters (for ref see Eccles Ito and Szentagothai 1967) Hence the cerebellar inhibition of the defence reaction studied here may have been caused by stimulation of such afferents or by an activation of Purkinje fibres On the basis of the present experiments alone it is impossible to exclude any of these alternatives This is especially true since little is known about the cerebellar afferents responsible for the dual action of cortical stimulation Provided that activation of Purkinje fibres cause the inhibition of the defence reaction this may be accomplished by at least one of the following three mechanisms An inhibition of neurons in the sympathetic vasodilator pathways, an inhibition of structures that facilitate the defence reaction or a disinhibition of inhibitory structures, all acting by a direct action or *via* one or several interneurons The vasodilator pathways in the brain stem and the spinal medulla have cell bodies in the hypothalamus in the mesencephalon and in the lateral horns of the spinal cord (Lindgren 1955) An inhibition by means of the cerebellar influence may be exerted on one or more of these structures but it is less likely that it only affects the hypothalamic neurons as they, or their axons were directly stimulated by the hypothalamic electrodes in the present experiments

Most Purkinje fibres make synaptic connections in the cerebellar nuclei and the vermis region is known to project predominantly on the phylogenetically old fastigial nucleus Stimulation of this nucleus causes a generalized EEG arousal in intact animals (Moruzzi and Magoun 1949) Further sham rage can be facilitated from this cerebellar nucleus (Zanchetti and Zoccolini 1954) and certain findings suggest that some of its neurons may have a tonic activity (Eccles Ito and Szentagothai *e g* p 276 1967) It may be that this cerebellar nucleus exerts a tonic facilitatory influence on sham rage and the inhibitory effect on this activity pattern evoked by stimulation of the cerebellar cortex (Moruzzi 1947) would then be in conformity with the concept that the cerebellar cortex exerts an inhibitory influence on the underlying nuclei (Ito and Obata 1964) The effect of vermal stimulation on the defence reaction—induced by hypothalamic electric stimulation as in the present study—may possibly be explained in a similar way

There are no indications that the sympathetic vasodilator fibres exhibit any tonic activity under resting conditions (Lindgren 1955) neither do they participate in reflexes induced *via* the baroreceptors (Folkow and Uvnas 1948) However *a priori* it is possible that the impulse traffic along the central sympathetic vasodilator fibre connections can be counteracted by baroreceptor reflexes once the defence reaction

is induced Compatible with such a view is that distension of the carotid sinus can curb sham rage in decorticate cats (Bartorelli *et al* 1960) No evidence was found in the present experiments that baroreceptor reflexes exert any direct influnce on the sympathetic vasodilator fibre connections when these are activated from the hypothalamic defence area This emphasizes the suggestion that the baroreceptor reflexes act not in antagonism but in "hemodynamic synergism" with the cardiovascular defence reaction, when they are excited in the natural way (see also Djojosugito *et al* 1970 and Kylstra and Lisander 1970)

There is suggestive evidence that the hypothalamic defence area is involved in sham rage (Abrahams, Hilton and Zbrozyna 1960) A fundamental difference, however, must remain between the defence reaction, induced by topical hypothalamic stimulation and sham rage Since sham rage is triggered by external stimuli this reaction necessarily requires ascending pathways before the hypothalamic relay centre is reached and there are therefore several possible sites where an inhibition may take place This may explain why baroreceptor activation has no detectable effect on vasodilator fibre activity produced by D A stimulation, although intense baroreceptor excitation inhibits sham rage It is possible that the inhibitory influence of baroreceptor activation upon the sham rage response may be due to an impact on the ascending activating reticular formation The finding that powerful baroreceptor activation may induce signs of sleep (Bonvallet Dell and Hiebel 1954) and the known connections between buffer nerves on one hand and the medial reticular formation and the nucleus of the solitary tract on the other (Crill and Reis 1968) are in consonance with such an explanation.

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Studies of Blood-pressure Regulation
III. Dynamics of Arterial Blood Pressure on Carotid-sinus Nerve Stimulation

By

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Abstract

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An analysis of the properties of the blood pressure regulatory system has been made by means of carotid sinus nerve stimulation. *The method used involves analog simulation of the non linear properties of this reflex.* The analysis gives the gain frequency and the gain phase shift curves characterizing the system under study. A resonance frequency for the system is mostly found in the frequency range 0.03—0.06 Hz. Modulation of the control signal decreases the systemic bandwidth. Non linear simulation indicates that the mammalian blood pressure regulat … The afferent inform… pressure by means of … study are given. The system…

In previous studies (Öberg and Sjöstrand 1969 b) the regulatory properties of the carotid sinus reflex were studied in dogs by means of an analog simulation method. These studies gave qualitative information on the characteristics of the carotid sinus reflex on different types of electrical stimulation of the sinus nerve of both sides.

The dynamic properties of, for example, a technical control system can be examined by applying so called deterministic input signals to the regulatory system and measuring the response of the system to these signals. In this way it is possible *to obtain quantitative measures of the dynamic properties of the system.* This method has been applied to studies of several different biological regulatory systems including the carotid sinus reflex.

From this aspect the carotid sinus reflex can be studied by applying in principle deterministic signals in two forms, viz. intrasinusal pressure variations and electrical stimulation of the sinus nerves. Investigators including Scher and Young (1963), Levison *et al.* (1966), Stegemann and Müller Butow (1966), Spickler and Kezdi

(1967) and Christensen *et al* (1967) have applied intrasinusal pressure variations of different forms and studied the response in the form of systemic blood pressure changes and/or the impulse activity in the sinus nerve Correspondingly Allisen *et al* (1969) have studied the aortic receptor reflex Electrical stimulation of the sinus nerves has been performed by essentially the same method in experimental studies on animals by Warner (1958) Öberg and Sjostrand (1969 b) and Zerbst *et al* (1970)

Investigations of the mechano electrical properties of the baroreceptors by studies of the relationship between different intrasinusal pressure conditions and the sinus nerve activity have been reported by several authors (Bronk and Stella 1932 Landgren 1952 Christensen *et al* 1967, Spickler and Kezdi 1967 Katona *et al* 1968 and others) In some cases the studies have also included the influence of this type of stimulus on the systemic blood pressure In general however the sinus nerve activity has not been related in turn to the dynamics of the systemic blood pressure The production of reproducible baroreceptor activation by means of intrasinusal pressure variations can offer certain difficulties which is evident in a study of Katona *et al* (1968) among others

To be able to study dynamic relationships between the sinus nerve information and the systemic blood pressure it is necessary to have as stimuli direct and more reproducible activation of the afferent nerve fibers (constituting the sinus nerves) The method described in study II in this series permits the use of deterministic signals in the form of impulse trains for electrical stimulation of the sinus nerve

The aim of the present investigation was to study the effects on the circulatory system of electrical stimulation of the sinus nerves with determ nistic signals converted into pulse trains In this connection the carotid sinus reflex may be regarded as a two port network (black box) whose input signal (the intrasinusal pressure) produces frequency modulated signals in the carotid sinus nerve and whose output signal is the system c pressure All other receptors feedback chains and effector organs together with the experimental procedures are regarded as belonging to the two port network By this approach using a reduced system (lumped parameters) the system can be given a s mple mathematical form By this means conclusions can be drawn on the dynamic properties of the carotid sinus reflex under the simplified assumptions as given above Further the method used has distinct relevance to the technique (baropacing) which has come into use as a therapeutic method in certain forms of arterial hypertension—on the basis of data obtained by this technique in animal experiments the baropacer equipment can be made optimal

Methods

Exper mental an mals

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maintained at a constant rate of 33 mg/kg/hr (cf Arfors et al 1971) throughout the experiment The reasons for choosing chloralose as the anesthetic agent have been discussed previously (Öberg and Sjöstrand 1969 a, b) Although chloralose is generally considered to have little influence on the circulation, it can have some undesirable effects (Brown and Hilton 1935 Kirchheim and Gross 1970) The depth of anesthesia was checked at regular intervals and was maintained at such a level that the "back leg reflex" (to painful stimuli in the groin) was practically eliminated During the experimental part of the study a continuous intravenous infusion of blood isotonic dextran(6 %)-glucose(5 %) (Macrodex® glucose, Pharmacia Ltd [1] [illegible]

Recordings

The systemic blood pressure was measured with a Statham P23AC transducer via a catheter [illegible]

puter Intrasinusal pressures were measured with a separate transducer (Sanborn 267A) In some of the impulse function experiments the ECG (unipolar chest lead) was used as a trigger signal The recording equipment used in the previous studies was complemented by a memory oscilloscope (Tektronix 564)

Apparatus

The analog computer has been described previously but some new non linear properties have [illegible]

but also different time constants for the derivation function This was performed technically as follows

The zero crossing of the derivative is sensed with a comparator which controls two channels in which the positive and the negative derivative are amplified (the amplification can be varied independently) This differentiation method is referred to as 'split gain differentiation' (SGD) The previous and SGD methods of differentiation were easily interchangeable during the experiments

The model for these studies is illustrated in Fig 1 A In this figure H_2 is the transfer function for the blood pressure regulating system, with systemic blood pressure (systolic see Öberg and Sjostrand 1969 b) as the output and the information applied to the sinus nerves as the input, $H_1(s)$ is defined by the operator expression

$$H_1(s) = K_1 + \beta_1 \frac{C_1 s}{1 + \tau_1 s} + \beta_2 \frac{C_2 s}{1 + \tau_2 s}$$

where

K_1 = proportional gain factor

C_1 = positive rate sensitivity factor

C_2 = negative rate sensitivity factor

$\tau_{1,2}$ = time constants

s = Laplace operator

where $\left.\begin{array}{l}\beta_1 = 1\\ \beta_2 = 0\end{array}\right\} x > 0$

$\left.\begin{array}{l}\beta_1 = 0\\ \beta_2 = 1\end{array}\right\} x < 0$

The simulator with the transfer function H_1 forms a regulatory system with H_2 as shown in Fig 1 A

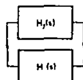
The experiments in the frequency domain were performed with the analog computer as described above as encoder The general principle of the model is described in Fig 1 B The transfer function was analogous with the above in certain qualitative experiments in the frequency domain but had the form

$$H_1^1(s) = K_1 + \frac{C_1 s}{1 + \tau_1 s}$$

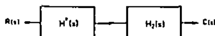
[1] Macrodex was kindly supplied by Pharmacia Ltd , Uppsala for which we express our thanks.

SYSTEMS ANALYSIS MODELS

A Closed loop SGD stud es



B Frequency domain stud es (descr bing function)



C T me doma n stud es (mpulse function)

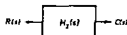


Fig 1 Schematic experimental models of the studies described in the text

receptor activity

In order to measure and record the instantaneous stimulation frequency a D/A converter was used In the impulse function studies the stimulation impulses were also counted on a Racal 835 counter (Racal Instruments Ltd , England)

Electrodes

The same type of stimulation electrodes as before were used *i e* two metal bands 1 mm broad and 0 1 mm thick (at a mutual distance of 3 5 mm) These surrounded the sinus nerve like snares The diameter of this snare was usually about 4 mm after its application so that good contact with the nerve bundle was achieved

In order to attain a maximal stimulation effect (electrical activation of a maximal number of nerve fibers) the electrode impedances should be kept as low as possible The results of a study of the impedance conditions in electrodes of different materials but with the same geometrical form as those described above are given in Fig 2 This study comprised both animal and model experiments The measurements were performed with a Hewlett Packard Vector Impedance Meter 4800 A (Hewlett Packard USA) For the series of animal experiments the borderlines within which all measurement results can be contained are given both for the magnitude of the impedance and for the phase angle The model studies were conducted in an electrolyte bath with electrode diameters of 4 mm As can be seen in the figure platinized platinum electrodes showed the lowest magnitude of impedance together with a very low capacitive reactance in both the animal and model stud es and this type of electrode was therefore used throughout in the stud es reported in this paper The data for the stimulation pulses were the same as in the previous investigation

Experimental procedure

In the previous study it was demonstrated that different properties of the model gave different

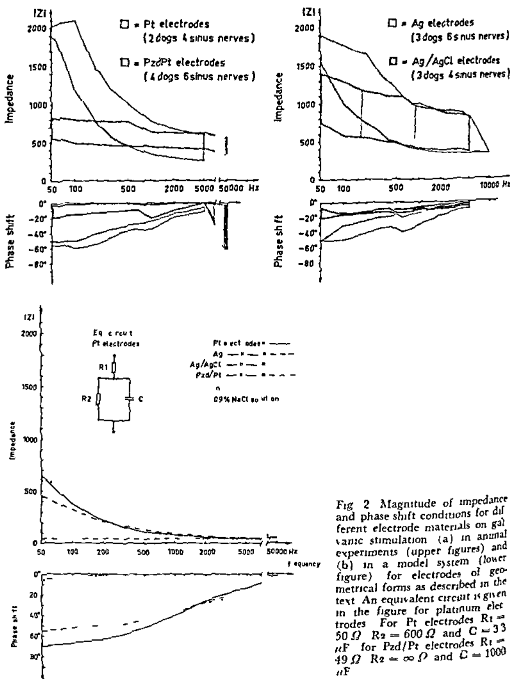


Fig 2 Magnitude of impedance and phase shift conditions for different electrode materials on galvanic stimulation (a) in animal experiments (upper figures) and (b) in a model system (lower figure) for electrodes of geometrical forms as described in the text An equivalent circuit is given in the figure for platinum electrodes For Pt electrodes $R_1 = 50\ \Omega$ $R_2 = 600\ \Omega$ and $C = 33\ \mu F$ for Pzd/Pt electrodes $R_1 = 49\ \Omega$ $R_2 = \infty\ \Omega$ and $C = 1000\ \mu F$

types of systemic pressure response In this way the system could be given different degrees of stability by variation of the impulse coding It is of interest in this connection to study the influence of different gains in the positive and negative parts of the pressure derivative a property which has been demonstrated in pressure receptors *inter alia* By means of a simple change in the circuits this type of experiment (SGD) could be compared directly with these

which had the same gain for both derivative signs The normal regulatory response (Öberg

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excitation signal to the analog was then

$P_{stim} = \overline{P} + P_0 \sin 2\pi ft$

where f is the generator frequency,

$\overline{P}$ is the mean systemic 'pressure' and

Po is the amplitude of the pressure variations"

Some experiments in the frequency domain were performed with modulated signals The excitation signal in this case can be expressed as

$$P_{stim} = \overline{P} + P_0 \sin 2\pi ft + \frac{P_0}{10} \sin 2\pi f_0 t$$

where f = the 'carrier frequency

and fo = the modulation frequency

In the modulation studies f_0 was given the values 2, 6 and 10 Hz.

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systemic response is treated by numerical Fourier analysis (Manley 1945, Nielsen 1950) The amplitude of the fundamental component (P_{ASP}) in the Fourier series is used in the definition of the gain of the system

$$\text{Gain (dB)} = 20^{10}\log \frac{P_{ASP}}{P_0}$$

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short periods

impulse function), i e a period of stimulation so short that it ceases when the systemic response occurs The length of the stimulation interval was chosen in these impulse function studies such that a distinct systemic response was obtained but at the same time no longer than that the stimulation ceased before this systemic response appeared During the stimulation period a duty cycle of 0 33—6 7 %, in which the stimulation periods were 0 150—2 sec was chosen for the impulse train

Results

The qualitative results obtained in study II in this series were fully confirmed in the present study with regard to closed-loop simulation Different grades of stability of the system could thus be attained A purely proportional control gives in transient states damped oscillatory systemic responses An increase of the dynamic sensitivity gives a more damped response This change in stability is probably due to an alteration of the stimulation pulse distribution over each cardiac cycle

Experiments with SGD were performed in order to study the systemic effect of baroreceptor models with dynamic non linearities i e to elucidate the influence at the systemic level of the change in stimulation pulse distribution over the cardiac cycle The sensitivity to mean blood pressures (static factor K_1) the sensitivity to the positive derivative C_1 and the sensitivity to the negative derivative C were varied

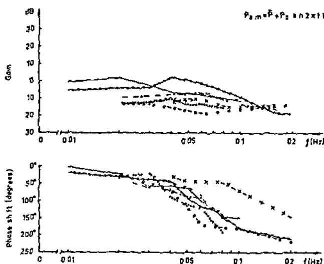


Fig 3 Describing function analysis in seven dogs

independently of one another in combinations in a large number of experiments (6 dogs) with transient changes in the systemic blood pressure No major essential differences as compared with the type of differentiation described previously were found A high negative rate sensitivity factor C_- (at moderate C_1 values) gave a damped return to the normal systemic pressure level after the inward course Similarly a low value for the static factor K_1 gave a correspondingly high systemic pressure level which was relatively independent of the values chosen for C_1 and C These results are in good agreement with those reported in study II, i e that K_1 is of importance for the systemic pressure (DC) level and C_1 for the stability of the system

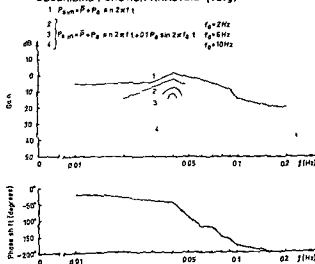


Fig 4 Describing function analysis in one dog (cf Fig 3) in the unmodulated case (curve 1) and at different degrees of modulation (curves 2—4) The gain of the system is related to the degree of modulation The phase shift is only shown for the unmodulated case

The frequency analysis in 7 of 9 animals in the series is shown in Fig 3 The gain of the system under the experimental conditions described under "experimental procedure" and according to the definition given above can be stated to be +4—(−18) dB within the frequency region of 0 01—0 2 Hz In general a maximal gain was observed within the frequency region 0 03—0 06 Hz, which is in full agreement with results obtained by Levison *et al* (1966) The exact slopes in the diagram on the high and low frequency sides are very difficult to determine with this method They are very steep, however, *i e* the frequency characteristics obtained as described above have the nature of an ideal band pass filter At the limits of the pass band, not only a low amplification but also non linear phenomena such as subharmonic oscillations are obtained, especially at the lower limit The phase shift characteristic shows a region of interest (the range 0 02—0 1 Hz) where the phase shift per decade is higher than in the lower and upper decades although the number of measurements in the latter cases are fewer

Fig 4 shows the analysis results from one of the seven animals on which modulation studies were performed The aim of the modulation of the signal of the function generator with another sinus signal of higher frequency was to simulate approximately the influence of the transient changes in blood pressure which occur at each pulse beat The figure is typical for the series

In the unmodulated case (1) there is agreement with Fig 3 On modulation (f_0 = 2 Hz) a limitation of the bandwidth of the blood pressure regulating system is obtained (curve 2), especially on the high frequency side of the maximal value of the amplification A reduction of the gain corresponding to about 5 dB is obtained In the modulated cases (3 4) the bandwidth decreases considerably, simultaneous with a decrease in gain (10 and 14 dB respectively), compared with the unmodulated case, *cf* Levison *et al* 1966) The phase shift is given only for the unmodulated case The frequency at which the gain is greatest is identical with the oscillatory frequency in the closed loop procedure

Non synchronized impulse functions studies were performed on 4 dogs (50 expts) with duty cycles of 1 2 3 and 4 % In all cases systemic responses were obtained for the different duty cycles The systemic responses to these non synchronized stimula-

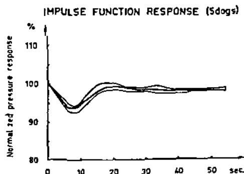


Fig 5 Implse function response to ECG-synchronized bursts (duty cycles 0 33—6 7 %) The envelope of the curve for 91 experiments limits the shaded area Mean value curve s indicated

tion bursts were more complex than those shown in Fig 5 for synchronized impulse bursts It is therefore difficult to formulate exactly the transfer function in these experiments An approximation for transfer functions of non oscillating systems can be obtained by a theory discussed by Paynter (1956) on an analogy between stochastic processes and monotone dynamic systems

The impulse function response has a time course $h(t)$, whose spatial distribution characterizes the studied system Given as a measure of this distribution are the first moment M_1 and the second central moment M_2 which are defined as

$$M_1 \triangleq T_m \cdot A = \int_0^{\infty} t \, h(t)\, dt$$

$$M_2 \triangleq T_s^2 \cdot A = \int_0^{\infty} (t - T_m)^2 h(t)\, dt$$

where

T_m = mean delay time

T_s = mean dispersion time

Here T_m and T_s are sensitive measures of the spatial distribution of the impulse function response and thus of the transfer function This also implies that impulse function tests may be useful as a clinical test in relation to baropacer therapy (see Interpretation)

The results of the analysis according to Paynter are given in Table I (data for one dog are given and also the ranges for 4 dogs)

TABLE I

Duty cycle	Tm sec	Tm range 4 dogs	Ts sec	Ts range 4 dogs
1 %	24 81	8 38—32 46	15 66	3 51—18 78
2 %	28 94	10 82—28 94	16 65	4 06—16 65
3 %	23 69	9 61—29 21	14 37	3 75—17 36
4 %	27 26	11 77—29 65	17 51	4 16—17 51

ECG synchronized impulse function studies were performed on 5 dogs (91 experiments) with duty cycles of 0 33, 0 67, 1 67, 3 3 and 6 7 % In all cases systemic responses were obtained for the different duty cycles but in comparison with the non synchronized studies the systemic responses were of a more 'linear' nature and can almost be characterized as a linear system of second order (*cf* Fig 5) The systemic responses for different duty cycles did not differ significantly from one another The transfer function H (see Fig 1 C) can as the result of these studies be expressed in the general form

$$H_1(s) = \frac{A(1 - e^{-as})}{s^2 + \alpha s + \gamma} + \varepsilon(s)$$

which characterizes the response in a system of the second order (which is disturbed by an impulse with amplitude A and duration a) α and γ are the conventional constants The correction term $\varepsilon(s)$ characterizes the long term adaptation of the blood pressure regulating system (*cf* Fig 5)

In Fig 5 a mean value curve (normalized scale) is shown for the experimental conditions described above The outer limits for the systemic responses in this case are also given in the figure (shaded area)

Interpretation

In studies I and II in the series the animals breathed spontaneously which in some cases caused pronounced vasomotor waves (Traube Hering type) These rendered more difficult the studies in the frequency domain By applying positive pressure ventilation with a small tidal volume and a high frequency (with moderately expanded lungs) these vasomotor waves were eliminated The respiratory cardiac arrythmia also ceased entirely The arterial pH pO and pCO were within the normal limits here and the spontaneous ventilation was completely eliminated (but on discontinuation of this positive pressure ventilation the spontaneous ventilation started again within 10—15 sec) The arterial blood pressure (systolic) was as a rule reduced by an average of 6 5 mm Hg on commencement of positive pressure ventilation the central venous pressure did not show any definite increase in the expiratory resting state during positive pressure ventilation. The elimination of the vasomotor waves can probably therefore be ascribed to the central communication between the respiratory and vasomotor centers and its modification in connection with the positive pressure ventilation The continuous expansion of the lungs will influence the stretch receptors in the lung parenchyma (constituting the inhibito inspiratory reflex the Hering Breuer reflex) and may lead reflectorically to an inhibition of the inspiratory center It can be assumed here that the spontaneous rhythmicity in the respiratory center is broken when the spontaneous ventilation is eliminated resulting in the absence of a rhythmical effect on the blood pressure Whether positive pressure ventilation means in turn an influence on the functional (dynamic) conditions of the blood pressure regulating system (*e g* the gain of the system) is difficult to decide The normal regulatory response (Öberg and Sjostrand 1969 a b) was however qualitatively (time domain) the same in this and the previous studies

In the experiments with SGD essentially no major differences were found compared with the other more simple type of dynamic sensitivity One possible explanation for this may be that the regulatory system by evolution has become more receptive to information from the baroreceptors during the systolic phase of the cardiac cycle *i e* a kind of sampled system might conceivably have arisen If this were so stimulation during diastole would be neglected by the vasomotor center which in fact is indicated by the experimental results (less sensitivity to negative derivatives)

When performing the frequency analysis one is faced with the compromise between a control signal with an amplitude so small that a linear mode of analysis can be used and a larger signal with a non linear systemic response With the small signal method difficulties were encountered in these experiments in such a small signal noise ratio was then obtained in the control variable that accurate deter-

minations were not possible The signal noise ratio was improved considerably in these experiments by, among other things, the fact that the respiratory influence on the systemic blood pressure was practically eliminated by high frequency positive pressure ventilation but there were still certain reflexogenic variations It was therefore chosen instead to work with a higher amplitude of the input signal and to apply quasilinearization in the theoretical treatment of the experimental material according to the method described above

The non linear systemic response to a control signal of high amplitude probably arises as a result of a dynamic non linearity in the smooth vascular muscle and may conceivably be caused among other things, by different velocities of the contraction and dilatation courses

As an example of the response of the system under study at extreme 'pressure amplitudes (P_0) in the simulator some additional studies were carried out With a sinus shaped input signal an output signal with a greater negative than positive derivative is obtained *i e* the decrease of the blood pressure is more rapid than the corresponding increase Distortion of this kind is more pronounced at low frequencies than at high and is a characteristic typical of certain biological systems—it is one of the part phenomena arising from the dynamic non linearity which is called unidirectional rate sensitivity This is demonstrated by Fig 6 where approximately sinus shaped frequency variations (generator frequencies 0 02 0 04 0 08 Hz) and *the corresponding blood pressure responses are shown The carotid sinus reflex* comprises in other words not only receptors with unidirectional rate sensitivity but also other functions (centers and effectors) with similar characteristics

In the light of the studies in the frequency domain (Fig 3 and 4) a comparison with the results obtained by Levison *et al* (1966) is of interest The variation of the gain with the frequency showed in principle the same course in both these investigations with a resonance frequency lying within the range 0 03—0 06 Hz

With regard to the phase shift of the system there is good agreement both quali

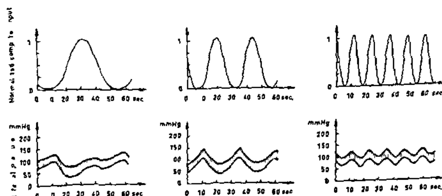


Fig 6 Sinus shaped stimulation frequency variations (generator frequencies 0 02 0 04 and 0 08 Hz) and the related blood pressure response in one dog (see the text) Note the variations in the pulse pressure indicated in the blood pressure recording

tatively and quantitatively Especially interesting is the interval with greater phase shift per frequency unit which occurs within the range mentioned above The good conformity between the two basically different methods (intrasinusal pressure variations and electrical stimulation of the sinus nerves) of studying the frequency dependence of the carotid sinus reflex indicates that the two methods for frequency analysis of the low pass system in question here are more or less equivalent The details of the afferent sinus nerve activity can be assumed to differ in these two methods In the light of this comparison (on a limited experimental material) details in the sinus nerve information do not seem on the other hand to have decisive importance on the systemic level This assumption is supported by the fact that in the impulse function studies (synchronized and non synchronized), essentially the same systemic response was obtained to a large variation in the number of stimulation pulses (5—800) Of importance in this connection are recently reported studies (McKean *et al* 1970) in which the frequency was found to be the information carrier in an impulse train

It is evident from Fig 3 that in all cases the gain is less than unity at a phase lag of 180° which means a stable system Fig 4 provides clear confirmation of the finding in the time domain in study II that modulation gives increased stability of the system This observation can obviously be due to the fact that the bandwidth of the system greatly decreases on modulation probably due to an effective inhibition of the vasomotor center At the highest degree of modulation the bandwidth was so small that the systemic response could only be obtained at one single frequency viz the resonance frequency It is important to take this latter finding in consideration when working out the specifications for a baropacer Among other things it means that the question of whether constant or intermittent (alternatively modulated) electrical stimulation of the sinus nerves should be used in baropacer therapy (*cf* Parsonnet *et al* 1969) can be answered Intermittent stimulation is to be preferred on account of its greater blood pressure reducing capacity and its bandwidth limiting effect the latter effect is especially relevant for a baropacer since by this means it will stabilize the system This phenomenon can also be regarded as a consequence of the unidirectional rate sensitivity of the system where one of the characteristics is a rectification process which gives rise to a larger decrease of the DC level Further support for these assumptions has been gained from acute experiments in the dog and this will be reported in a following paper The decrease of the gain as well as the bandwidth reduction on modulation can be considered to be due to non linearity of the system

In choosing the stimulation frequencies in the impulse function studies the lowest frequency was chosen so that it gave a clear blood pressure response and the highest so that it could be assumed that the momentary stimulation frequency never exceeded the corresponding refractory period of the nerve fibers The reason for the response of higher order obtained in the non synchronized impulse function studies was probably the interaction between the artificially and endogenously generated information flows A further example of such interaction has been reported previous-

ly (Öberg and Sjöstrand 1970) In the ECG-synchronized impulse function studies (see Fig 5), practically linear responses were obtained, according to a previously defined operator expression. In this expression e^{-sT} represents the transport delay that occurs in most biological regulatory systems The reason for the poor differentiation between duty cycles of different magnitudes lies in the low pass properties of the system as discussed above

In the asynchronized impulse function experiments it appeared that the values of T_m and T_e had the same magnitude for the different duty cycles in one and the same experimental animal In different animals relatively large differences in the T_m and T_e values were evident Table I gives the values for one experimental animal and also the range of T_m and T_e values in a series of four dogs The relatively wide range in T_m and T_e can probably be explained by different systemic gains and uncontrollable variations in the experimental conditions (see Fig 3)

The Paynter method of giving an approximative expression for the transfer function of a high ordered system in terms of T_m and T_e may be useful to follow the functional state of a hypertensive patient with implanted baropacer electrodes on a chronic basis The T_m and T_e values can also give guidance in determining the optimal baropacer settings in each individual case

To summarize *the results obtained mean from a physiological aspect* that

the afferent information of the baroreceptor reflex can be assumed to be bandwidth limiting because of its pulsating nature

the system during its development up to the mammalian series has partly acquired the character of a sampled regulatory system,

the stated transfer functions characterize the system,

and *from a baropacer aspect* that

pulsed stimulation bursts synchronous with the endogenous baroreceptor information give a greater percentual blood pressure reduction and at the same time stabilization by bandwidth limitation

the impedance conditions of the stimulation electrodes are important for effective nerve stimulation

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The Effect of Lactate in Canine Subcutaneous Adipose Tissue in Situ[1]

By

BERTIL B FREDHOLM

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Abstract

FREDHOLM, B B *The effect of lactate in canine subcutaneous adipose tissue in situ* Acta physiol scand 1971 81 110—123

Na L(+) lactate and Na pyruvate were administered by intraarterial infusion in canine subcutaneous adipose tissue, perfused with the dogs own blood either at a constant rate from a reservoir or by autoperfusion The ... glucose concentration Similarly t... arterial concentrations the latter ... below 5 mM and Na D(—) lacta... glycerol upon nerve stimulation (4 cps for 5 to 10 min) On the other hand Na L(+) lactate above 10 mM caused a 70 per cent ... ing the glycerol release Na pyruvat... increased the FFA release Neither ... take Na lactate was not vasoactive, ...

It is concluded that lactate in concentrations occurring *e g* during muscular exercise and shock is capable of significantly depressing the rate of FFA release upon nerve stimulation by increasing the rate of re esterification The finding that lactate and pyruvate had opposite effects on esterification indicates a role of the cytoplasmatic NADH/NAD ratio in determining the rate of esterification

Marked increases in blood lactate levels are seen in several conditions such as severe exercise and hemorrhagic shock (Åstrand and Rodahl 1970 Kovach 1955) An increased production of lactate following the administration of catecholamines (especially adrenaline) has also been demonstrated in many species (see Hagen 1967) The lactate production may be considered to be due to an inbalance between glycolysis and oxidative capacity (Keul Doll and Keppler 1969), caused by a limitation of pyruvate oxidation (Holloszy 1967) and/or to the mitochondrial oxidation of cytoplasmatic NADH (Boxer and Devlin 1961 Alpert 1965) Fatty acids may interfere with the first process (Garland Newsholme and Randle 1964) and the latter might be deficient in adipose tissue (Schmidt and Katz 1969)

[1] Part of these results were presented at the Meeting of Scand Physiol Soc in Gothenburg 1969 (Fredholm Acta physiol scand suppl 330 p 77 1969)

The infusion of sodium lactate, producing blood lactate levels corresponding to those seen in exercise, caused a decrease in plasma FFA (Issekutz and Miller 1962) This was shown to be due to an inhibition of FFA mobilization (Miller, Issekutz and Rodahl 1963, Miller *et al* 1964)

Studies by Bjorntorp (1965) on rat epididymal adipose tissue *in vitro* demonstrated a significantly lower glycerol production in tissues incubated in 3 mM lactate, as well as a significant inhibition of lipolytic activity in extracts from lactate treated fat pads

The present study was undertaken to see if lactate could inhibit the mobilization of FFA from isolated canine subcutaneous adipose tissue particularly under conditions of sympathetic nerve stimulation (Rosell 1966) The effect of graded infusions of lactate on both glycerol and FFA release was studied in an attempt to ascertain whether the action is primarily on lipolysis (Bjorntorp 1965) or re esterification (Miller *et al* 1964) In order to further clarify the mechanism of action the effect of pyruvate administration was also studied

Methods

Materials and experimental procedure

The experiments were conducted on 29 female mongrel dogs weighing between 7 and 24 kg Subcutaneous adipose tissue situated on the right side of the abdominal midline and medial to the inguinal ligament was isolated as described by Rosell (1966) The tissue weight ranged between 12 and 58 g (mean 34 g) The mixed nerve to the adipose tissue was cut at the level

determined

In 8 expts the drop counter interrupted a cannula between the ipsilateral femoral artery and the artery to the adipose tissue The venous outflow was returned to the systemic circulation via a femoral vein Samples of the venous effluent from adipose tissue for analysis of metabolites were drawn by means of a three way stop cock arrangement

Perfusion pressure and/or systemic arterial blood pressure were measured with Statham pressure transducers and recorded together with the blood flow on a Grass model 5B polygraph

constant flow experiments and generally discontinuous in free flow experiments Samples were drawn before during and after the nerve stimulation (5 to 10 min) both during infusion of the test solution and during the control infusion of saline

In constant flow experiments arterial blood samples were drawn from the reservoir one immediately before one immediately after the experiment Occasionally blood samples were drawn at intermediate times also In the free flow experiments blood was drawn from the femoral artery at 15 to 30 min intervals In all experiments an aliquote of the infusion solution (generally 0 1 ml) was added to an aliquote of arterial blood (generally 5 ml) By subjecting

[illegible] od samples the arterial con[illegible] calculated The uptake o[illegible] lifferences by the blood o[illegible] plasma flow in ml/min 100 g

Chemical determinations

FFA were determined titrimetrically according to Dole (1956) as modified by Trout *et al* (1960)

Glycerol was determined by enzymatic analysis (Laurell and Tibbling 1966) Glucose was analyzed enzymatically using commercially obtainable reagent (Glox, Kebo) Lactate and pyruvate were enzymatically assayed using test kits from Boehringer & Sohn Mannheim

Chemicals

L(+) lactic acid (17 % D(—) lactic acid contamination) and Na pyruvate (99 7 % pure) was obtained from Fluka AG Basel L(+) lactic acid (1 2 per cent D(—) lactic acid) D(—) lactic acid (1 3 per cent (L(+) lactic acid) from Sigma St Louis, Glox reagent and L-cystein from Kebo Stockholm nicotine adenine dinucleotide glycerol 1 phosphate de hydrogenase glycerolkinase from Boehringer & Sohn Mannheim.

Statistical analysis

Statistical analysis was performed as described by Sokal and Rohlf (1969) Except where else where indicated the statistical hypothesis tested was that the net release or uptake of a metabolite during the infusion of a test solution is identical to the parameter during the in fusion of saline in the same experiment (t test for paired variates)

Results

Section A Methodological studies

Changes in the arterial blood composition

The composition of arterial blood as well as the metabolism during basal conditions (see below) was studied in a total of 72 dogs, results from which have been partly presented elsewhere (Fredholm and Rosell 1968, Fredholm and Rosell 1970) In the free flow experiments the arterial concentration of the metabolites investigated (glycerol FFA glucose lactate pyruvate) varied somewhat This was probably due to a multitude of causes such as differences in the depth of anesthesia blood loss due to sampling and the administration of the test solution to the animal via the venous return from adipose tissue These fluctuations could be accounted for by frequent arterial samples

In order to minimize fluctuations in the composition of arterial blood constant flow perfusion from a reservoir was used in the majority of the experiments The hematocrit (35 range 23—55) glycerol (0 08, 0 004—0 28 mM) and FFA (0 28 0 04—0 58 mM) did not change in the reservoir in the course of the experiment On the other hand the glucose concentration in the reservoir (5 33 + 0 31 mM n= 40 in blood, 6 28 ± 0 24 mM n = 33 in plasma) decreased during the course of the experiment The magnitude of this decrease was associated with the initial glucose concentration ($p < 0\,01$ by the corner test of association) but correlated poorly with the amount of time in the reservoir The arterial lactate concentration was always higher at the end (2 29 ± 0 38 mM) than at the beginning (1 46 ± 0 13 mM) of the experiment This increase was poorly associated with the initial lactate concentra

tion and with the magnitude of the simultaneous decrease in glucose but well correlated with the time in the reservoir (Rank correlation coefficient 0 95, n = 38) A kinetic analysis of these changes in the arterial blood composition was not performed but the changes with time in the individual experiments could be well fitted to a straight line Thus the arterial concentration of lactate and glucose at any given time during the experiment was determined by linear intrapolation between the samples taken at the start and at the end of the experiment

Comparison between free flow and constant flow perfusion

In order to determine whether or not there were any differences between the two experimental techniques an analysis of variance was carried out No significant differences with regard to any of the metabolic parameters was found The results are therefore presented together

Reproducibility of the nerve stimulation

Statistical testing involved the comparison of two nerve stimulations one performed during the infusion of saline the other during the infusion of an organic anion In order to check the reproducibility of the nerve stimulation 6 expts were performed where two nerve stimulations in series during saline infusion were compared The results are given in Table I There were no differences between the two periods

Comments to section A

Two different perfusion techniques, which are probably not subject to exactly the same errors were used in order to eliminate some of the methodological hazards They gave essentially identical results Treatments and control periods have been subject to a pseudorandomization, *i e* the control period was not always preceding the experimental period in time This was possible since the response to nerve stimulation was essentially the same before and after an infusion of Na L(+) lactate (Fig 1) The reproducibility of the nerve stimulation was considered sufficiently good for the purposes of the present investigation

TABLE I Comparison of the effects of repeated nerve stimulations in the same dogs on glycerol and FFA release (μmoles/100 g tissue)

Exp no	Glycerol release		FFA Release	
	Stim I	*Stim II*	*Stim I*	*Stim II*
1	17 2	19 0	8 0	8 9
2	—	—	5 1	6 0
3	24 9	29 3	1 2	4 7
4	12 3	11 7	16 1	11 4
5	11 0	8 2	9 0	6 9
6	18 9	19 3	—	—
Mean	16 9	17 5	7 9	7 6
S E M	2 5	3 6	2 5	1 2

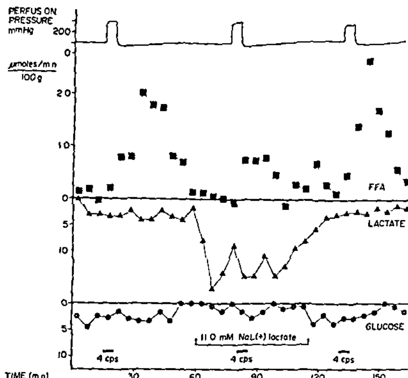


Fig 1 The effect of increasing the arterial lactate concentration by about 11 mM Dog 10 kg anesthetized with 25 mg/kg Nembutal i v Adipose tissue weight 28 g Perfusion with defibrinated blood (Hct 30 0) at a constant rate (5 7 ml/min/100 g)

Section B Effects of lactate and pyruvate

Metabolism during basal conditions in adipose tissue during saline infusion

During basal conditions in 72 dogs glycerol release averaged 0 24 µmoles/min/100 g adipose tissue (range 0 02—0 71) No relationship between glycerol release rate and the arterial glycerol concentration could be found The basal FFA release averaged 0 12 µmoles/min/100 g adipose tissue (range −0 96—1 25) As with glycerol no relationship between the arterial concentration of the metabolite and the net release was detected On the other hand the glucose uptake was strongly dependent on the arterial glucose concentration as is seen in Table II

TABLE II Glucose uptake at different arterial glucose concentrations

Arterial glucose mM	Glucose uptake[1] µmoles/min/100 g	n[2]
2—3 5	1 82 (0 04—4 92)	120
3 5—5	3 45 (0 56—9 75)	120
> 5	3 79 (0 28—14 11)	240

[1] mean and range
[2] 6 duplicate or quadruplicate determinations from each dog

The effect of nerve stimulation

The stimulation of the mixed nerve to the adipose tissue with 4 cps caused the release of glycerol (16 1±1 70 μmoles per 100 g adipose tissue n=39) and FFA (13 6±2 25 μmoles of FFA per 100 g tissue n=39) The nerve stimulation did not affect the glucose uptake significantly No clearcut differences in the uptake or release of lactate was observed before, during and after the nerve stimulation Nor were any significant effects on the pyruvate uptake or release detected

Uptake of lactate and pyruvate by adipose tissue

The rate of pyruvate as well as lactate uptake was found to be strongly dependent upon the arterial concentration of the respective anion In both cases this dependence was found to be well described (p<0 01) by a linear regression It can be noted that the slopes of the regression lines (see Fig 2) are dissimilar Thus the rate of pyruvate uptake apparently increases more rapidly for a given increment in arterial concentration of the anion than does the lactate uptake Furthermore, both regression lines intercept with the abscissa at positive values of the arterial anion concentrations which are in the concentration range normally found in arterial blood during resting conditions

Effects of L(+) lactate

Increasing the arterial lactate concentration by approximately 2 mM had no clearcut effect on FFA and glycerol release or on glucose uptake (Table III)

A further increase of 5 to 7 mM likewise failed to change the amount of FFA and glycerol released upon nerve stimulation However, 10—14 mM lactate in arterial

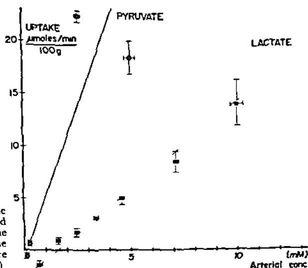


Fig 2 Relationship between the uptake of lactate (n = 232) and pyruvate (n = 108) and the arterial concentration of the anion Both regression lines are statistically significant (p<0 01)

TABLE III Effects of Na L(+) lactate on glycerol, FFA, glucose, lactate and pyruvate

Exp no	Glycerol release Basal I	II	Stim I	II	FFA release Basal I	II	Stim I	II	Glucose uptake I	II	Lactate uptake I	II	Pyruvate uptake I	II
2 mM lactate														
1	28	04	7 6	6 0	23	04	10 8	10 5	165	193	218	407		
2	06	16	3 8	3 5	10	08	4 7	2 5	154	204	200	395		
3	—	—	—	—	—55	—54	5 4	7 2	381	304	87	428		
4	20	12	26 0	31 8	—	—	—	—	246	240	149	188		
Mean	18	11	12 5	13 8	—07	—14	7 0	6 7	236	235	163	354		
S E M	06	04	6 9	9 0	24	20	1 9	2 3	52	25	29	56		
5—7 mM lactate														
1	20	11	28 3	37 8	—79	—59	7 8	4 1			169	1 048		
2	12	10	8 4	9 1	04	—02	11 5	7 5	110	225	120	745		
3	53	56	8 5	6 6	35	34	3 3	3 1	201	236	—10	301		
4	21	05	7 4	7 7	—16	—18	0 2	2 5	151	99	20	480		
5	36	50	7 2	6 1	13	13	2 9	3 8	243	228	447	1 235	—4	2
6	22	26	12 6	9 3	08	06	13 9	9 4	225	236	38	163		
Mean	27	26	12 4	12 8	—06	—04	6 6	5 1	197	205	131	662	—4	2
S E M	06	09	3 4	5 0	16	13	2 2	1 1	12	27	69	173		
9—11 mM lactate														
1	59	35	4 5	5 4	—96	—79	29 6	9 0	684	398	84	1 308		
2	25	22	6 9	5 7	—16	—01	11 0	2 9	441	578	220	934		
3	—	—	—	—	10	01	37 8	19 6	169	65	105	406		
4	11	13	37 8	26 4	—79	—69	8 5	3 3	238	258	—	—		
5	42	42	12 1	15 3	—42	—38	20 5	3 0	330	284	27	388		
6	07	14	25 8	9 8	—10	—25	23 7	0 7	484	412	375	1 463	23	23
Mean	29	25	17 5	12 5	—39	—35	21 8	6 4	391	333	162	900	23	23
S E M	10	06	6 3	3 9	17	14	4 5	2 9	76	71	62	223		
16—19 mM lactate														
1	59	31	12 2	15 1	—95	—84	18 7	10 8	691	468	110	2 593		
2	25	44	6 9	5 3	—16	—10	11 0	1 9	441	546	220	2 119		
3	28	15	11 8	5 3	—26	—29	16 4	4 8	285	252	134	563		
4	43	43	42 6	11 6	—25	—16	86 0	3 3	380	467	160	2 514		
5	50	15	10 0	14 0	—50	—50	4 6	1 7	516	620	90	1 146	—50	—4[illegible]
Mean	41	30	16 7	10 3	—43	—38	27 4	4 5	463	471	143	1 787	—50	—4[illegible]
S E M	06	06	6 6	2 1	14	13	14 9	1 7	68	65	23	400		

Basal glycerol and FFA release as μmoles/min/100 g
Stimulated glycerol and FFA release μmoles/100 g
Glucose lactate and pyruvate uptake μmoles/hour/100 g

Period I — saline infusion
Period II — lactate infusion

blood produced a clearcut inhibition of the release of FFA upon nerve stimulation from subcutaneous adipose tissue (Fig 1) A tendency towards a decreased glycerol release—indicating an inhibition of lipase—was also noticed (Table III) This was not significant, however The glucose uptake was not significantly changed by this concentration of lactate

The highest lactate concentrations ranging between 17 and 21 mM gave essentially the same results (Table III)

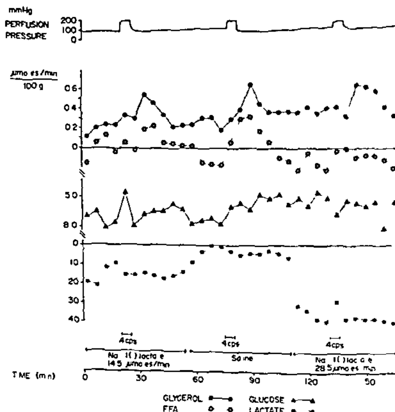


Fig 3 The effect of increasing the arterial lactate concentration by about 9 and 17 mM respectively Dog 9 kg Anesthetized with Nembutal 25 mg/kg iv Adipose tissue weight 38 g Perfusion with defibrinated blood at constant rate (Hct 27, 4 2 ml/min/100 g)

A typical experiment is illustrated in Fig 3 Two infusions of Na lactate were given separated by an infusion of saline of the same duration The arterial lactate concentration was increased by approximately 9 and 17 mM respectively It is seen that the amount of FFA released upon nerve stimulation is higher during the saline infusion than the amount released during either lactate infusion Yet the glycerol release was if anything higher during the lactate infusion

Effects of pyruvate

In order to elucidate the mechanism behind the effects of lactate pyruvate in high amounts increasing the blood level of pyruvate to about 5 mM was administered to subcutaneous adipose tissue in four dogs (Table IV) Pyruvate had a different effect on FFA release upon nerve stimulation than lactate Thus the amount of FFA release increased even though the glycerol release was lowered ($p < 0.05$) The ratio between FFA and glycerol release increased significantly ($p < 0.05$) indicating a decreased re esterification

TABLE IV The effect of Na pyruvate (4—6 mM) on the release of FFA and glycerol and the uptake of glucose lactate and pyruvate

Exp	Glycerol release				FFA release				Glucose		Lactate		Pyruvate	
no	Basal		Stim		Basal		Stim		uptake		uptake		uptake	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II
1	20	22	18 6	15 7	14	02	5 4	8 6	204	144	53	47	— 8	257
2	45	25	49 1	33 5	20	20	12 5	12 3	390	258	29	— 13	— 5	1 922
3	24	18	16 5	15 5	— 08	— 10	9 5	28 2	319	261	30	0	—25	474
4	43	31	18 0	14 1	03	00	28 0	21 8	118	156	—41	— 97	— 8	1 314
5	24	11	10 0	5 2	— 02	— 13	11 0	10 6	129	140	—44	—102	— 6	693
Mean	31	21	22 5	16 2	05	00	13 5	16 3	232	192	5	— 33	—10	931
S E M	06	04	6 8	5 2	05	07	3 8	3 7	53	28	20	29	4	304

Basal FFA and glycerol release μmoles/min/100 g
Stimulated FFA and glycerol release μmoles/100 g
Glucose lactate and pyruvate release μmoles/hour/100 g

Period I — saline infusion
Period II — pyruvate infusion.

Pyruvate infusion decreased the net lactate uptake by adipose tissue (Table IV) probably indicating the formation of lactate from pyruvate These effects are demonstrated in Fig 4 A reduction in the perfusion pressure following pyruvate administration indicating a decreased vascular resistance is also seen

The effects of lactate and pyruvate infusions are summarized in Fig 5 The amounts of FFA and glycerol released during an infusion of lactate or pyruvate is given in per cent of the amounts released during a stimulation while saline is infused The value plotted is the mean of the per centages in the individual experiments

Effects of D(—) lactate

The Na(+) lactate preparations used in the present study were to variable degree contaminated by its optical isomer (see Methods) In order to investigate whether

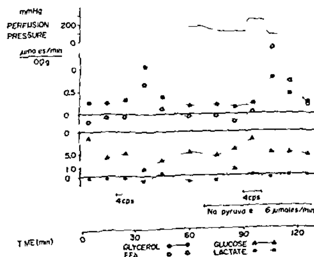


Fig 4 The effect of increasing the arterial pyruvate concentration by 4 mM Dog 12 kg anesthetized with sodium pentobarbital 25 mg/kg iv Adipose tissue weight 25 g Perfusion with defibrinated blood at a constant rate (Hct 35, 6 0 ml/min/100 g)

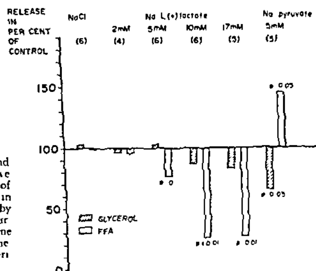


Fig 5 The release of FFA and glycerol by sympathetic nerve stimulation during the infusion of saline, lactate and pyruvate in per cent of the release caused by a similar nerve stimulation during saline infusion in the same animal Figures within parenthesis denotes the number of experiments

D(—) lactate contributed significantly to the effects reported above the experiments reported in Table V were performed No significant responses to Na D(—) lactate (10—12 mM) were detected Thus the effects of lactate are in all probability due to the dextrorotatory isomer

Vascular effects

Adipose tissue vascular resistance, defined as the ratio between perfusion pressure or mean systemic arterial blood pressure in mm Hg and the blood flow in ml/min/100 g varied considerably among preparations (9 4—114 6 resistance units) The stimulation of sympathetic nerves always increased the resistance (246±32 per cent)

TABLE V The effect of Na D (—) lactate (about 10 mM) on the relase of FFA and glycerol before and after nerve stimulation

Exp no	Glycerol release Basal I	II	Stim I	II	FFA release Basal I	II	Stim I	II
1	28	10	7 2	7 0	— 12	— 14	4 0	5 2
2	02	11	2 0	7 1	— 16	— 12	7 2	4 8
3	37	20	18 2	37 6	— 12	— 25	45 3	31 3
4	07	08	27 0	18 1	— 07	— 12	15 1	16 4
Mean	18	12	13 6	17 4	— 12	— 16	17 9	14 4
S E.M	08	03	5 6	7 2	02	03	9 4	6 2

Basal FFA and glycerol release μmoles/min/100 g
Stimulated FFA and glycerol release μmoles/100 g

The infusion of the lactate generally caused no change in the vascular resistance but pyruvate produced a definite reduction in vascular resistance ($p<0.05$). There were no significant differences in the vascular response to nerve stimulation during infusion of anion and saline.

Comments to section B

The present experiments have demonstrated a disappearance of lactate and pyruvate from blood during the passage through adipose tissue. The fact that the infusion of lactate was not accompanied by a large increase in venous pyruvate, and likewise a pyruvate infusion not by large increases in venous lactate concentration (Table III) might indicate that the anions were in fact extracted by the adipose tissue and not merely converted in the blood stream.

It is possible that a major part of the uptake of lactate and pyruvate by adipose tissue is an accumulation of the anions in the extracellular space. However, the fact that the cessation of a lactate infusion did not lead to a large net release of lactate from adipose tissue makes this possibility less likely. Probably therefore a major part of the anion disappearance during a passage through adipose tissue reflects the uptake by adipocytes.

Stimulation of the sympathetic nerves during saline infusion released approximately equimolar amounts of FFA and glycerol. Assuming a negligible partial hydrolysis under these circumstances as well as a negligible utilization of glycerol this would mean that about two thirds of the fatty acids formed in adipose tissue are re-esterified (see Steinberg and Vaughan 1965). When increasing the lactate concentration by 9 mM or more the ratio between the output of FFA and glycerol was decreased. This would mean that in this case re-esterification has increased. Such a mechanism of action of lactate was also suggested by Miller *et al* (1964).

It is generally accepted that the glycerol moiety for triglyceride synthesis is derived from α-glycerolphosphate (α GP) from dihydroxyacetonephosphate (DHAP) in the Embden-Meyerhof pathway. Since the cytoplasmatic α GP/DHAP ratio is dependent upon the cytoplasmatic NADH/NAD ratio and since lactate and pyruvate had opposite effects on the release of FFA and glycerol a speculative mechanism of action of lactate can be formulated. When lactate is metabolized by the cells it is transformed to pyruvate in which process NADH is formed. This NADH might shift the α GP $\longleftrightarrow$ DHAP equilibrium to the left thereby furnishing more glycerol equivalents for triglyceride synthesis as suggested by Miller *et al* (1964).

In a recent study on the metabolism of lactate and pyruvate in epididymal fat pads of the rat Schmidt and Katz (1969) found evidence to indicate that hydrogen equivalents from lactate cannot be transferred into the mitochondria. These results support the hypothesis that changes in the rate of lactate utilization should influence the extramitochondrial NADH/NAD ratio. These authors also pointed out that a high re-esterification of fatty acids might be an adaptation to the need to reoxidize excess cytoplasmic NADH.

Miller *et al* (1964) found that the infusion of Na pyruvate to intact dogs had the

same effect on plasma FFA as a infusion of Na lactate This is in apparent contradiction to the present results and to the proposed mechanism However the authors pointed out that it is likely that a major part of the pyruvate infused into the intact dog is transformed to lactate in blood and tissues Such conversion was not observed in the present study

Miller *et al* (1964) considered the possibility that an increased blood flow through adipose tissue might be the explanation of their finding that lactate inhibited FFA release and presented circumstantial evidence against it This conclusion is supported by the present direct demonstration that lactate is not vasoactive in adipose tissue The poor vascular activity of the lactate ion has been demonstrated repeatedly in other vascular beds (see Haddy and Scott 1968) The mild vasoactivity of pyruvate is also well known

General discussion

The present results have demonstrated that arterial lactate concentrations above 10 mM reduce the output of FFA during sympathetic nerve stimulation in canine subcutaneous adipose tissue It has been shown that lactate infusion to the intact dog leads to decreased plasma FFA levels and FFA turnover (Miller *et al* 1964) Bjorntorp (1965) found that 3 mM lactate added to the incubation fluid inhibits lipolytic activity in the epididymal fat pad of the rat The difference between the latter study and the present one could perhaps be accounted for by species differences as well as by the large diffferences in technique On the other hand the results of Miller *et al* (1964) and the present ones are completely compatible

Muscular exercise of maximal or almost maximal intensity of short duration can lead to lactate levels in blood of 20 mM or more (*e g* Hermansen and Saltin 1966) It is known that the energy for such exercise derives from oxidation of carbohydrate rather than fat (Christensen and Hansen 1939 Keul Doll and Keppler 1969) Mobilization of depot fat thus serves little purpose yet it is known that the catecholamine levels in blood increase markedly with the severity of the muscular exercise (Haggendal Hartley and Saltin 1970) probably as a consequence of increased sympathetic nervous tone (Havel 1965 Rosell 1966 Havel 1968) and one would expect a large outflow of FFA during exercise Thus the lactate released from the heavily exercising muscle might serve to counteract the nervous stimulation of FFA release in a physiological condition where the need for metabolizable fat is not much increased

Lactate levels increase markedly in hemorrhagic shock (Kovach 1959) and arterial FFA levels are unchanged (Kovach *et al* 1970) or slightly increased (Halmagyi Irving and Varga 1968) in spite of a presumably high sympatho-adrenal activity (Walton *et al* 1959)

It has been shown repeatedly (see Flatt and Ball 1965 Landau and Katz 1965) that catecholamines increase lactate formation in epididymal fat pads *in vitro* Recently evidence for an increased lactate production in canine subcutaneous adipose tissue *in situ* during prolonged sympathetic nerve stimulation was presented (Fred

holm and Karlsson 1970) It is therefore possible that lactate formed within the adipose tissue could act to suppress FFA release

It is of interest in this context that catecholamines could increase lactate production not only by increasing glycogen breakdown (Hagen 1967), but also by increasing intracellular FFA levels (Weil, Ho and Altszuler 1965) Possibly therefore lactate might serve as a feed back regulator of FFA mobilization

The present studies have also revealed that the release of FFA can be profoundly altered although no gross changes in lipolytic activity—as measured by the rate of glycerol outflow—were detected This means that changes in the rate of re esterification in the adipose tissue could be an effective means of changing the mobilization of metabolizable fat The finding that the effects of lactate and pyruvate on re esterification are dissociated might furthermore point to a role of the intracellular red-ox potential in determining the rate of re esterification in adipose tissue

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Glucose Uptake at Rest and During Contraction in Isolated Dog Skeletal Muscle[1]

By

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Abstract

COSTIN J C, B SALTIN, N S SKINNER, JR. and G VASTAGH *Glucose uptake at rest and during contraction in isolated dog skeletal muscle* Acta physiol scand 1971 *81* 124—137

In an in situ isolated canine gracilis muscle preparation the a v difference for glucose oxygen and lactate has been studied at rest and during stimulation Both free flow (systemic blood flow) and constant flow perfusion (blood from reservoir) have been applied and in both sets of experiments the blood flow has been determined In the constant flow experiments no significant difference in the glucose uptake could be detected between stimulation as compared with rest. In the free flow experiments the same stimulation frequency produced a significant uptake of glucose ($\approx$ 20 µg glucose per g muscle and minute), which could account for approximately 10 per cent of the total energy output and 20—30 per cent of the total carbohydrate utilization It is suggested that the difference between the free flow and the constant flow experiments was due to the fact that in the free flow experiments where the muscle is perfused with the systemic blood flow some humoral factor is present which is necessary for the glucose to penetrate the cell barrier It was also possible to demonstrate a transient glucose uptake in the muscle in the constant flow experiments i e when the muscle was suddenly perfused with blood containing a high glucose concentration ($\approx$ 150 mg/100 ml blood

Carbohydrate metabolism is enhanced during muscular exercise and the degree to which this occurs in humans is related to the relative work level (for references see Hermansen Hultman and Saltin 1967) During prolonged heavy exercise diabetic and non-diabetic the concentrations of blood sugar have been shown to decrease (Dill, Edwards and Talbott 1932 Bøje 1936 Ingle, Nezamis and Morley 1951, Goldstein 1961 Larsson *et al* 1964 Young Pelligra and Adachi 1966) In addition. the concentration of plasma glucose has been reported to fall with muscular contraction in autoperfused dog and cat hind limbs (Huycke and Kruhøffer 1955,

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Chapler and Stainsby 1968) Since contracting skeletal muscle constitutes the major mass of tissue in all of the preparations used, these observations have led to the conclusion that the uptake of glucose in skeletal muscle is facilitated by contraction of this tissue Chapler and Stainsby (1968) have also recently shown a large uptake of glucose by isolated dog skeletal muscle during contraction and have concluded that the amount of glucose taken up by *in situ*, contracting skeletal muscle accounts for the major source of fuel

During prolonged heavy exercise in man approximately 3 g/min of carbohydrates are combusted (Hermansen *et al* 1967) If the major part of this glucose comes from extra muscular sources they will be exhausted within a short period of time Because of these contradictory results it was thought of value to further evaluate the uptake of glucose during skeletal muscle contraction in an isolated, *in situ* dog skeletal muscle

In addition, the influence of adrenalin and change in blood glucose concentrations on glucose uptake by inactive and active skeletal muscle was examined Finally, the effect of beta adrenergic blockade on glucose uptake and net glycogen depletion in contracting skeletal muscle was also evaluated

Methods

The right gracilis muscle of mongrel dogs of both sexes weighing 8 to 16 kg was used Anesthesia was produced with intravenously administered sodium pentobarbital (25 mg/kg) To

tubing and through this cannula blood pressure was measured and injections were made into the arterial blood flowing to the muscle The gracilis vein was cannulated with polyethylene tubing after all branches leading into it were tied so that the entire venous drainage from the muscle was directed through this cannula. The muscle was covered with mineral oil and its temperature was controlled and kept constant at around +38° C Blood flow was measured with a drop rate meter similar to the one described by Goldschmidt and Lindgren (1962), and pressure with a Statham P23b transducer

Constant Flow Perfusion with blood from reservoir was also employed and was accomplished by the method of Renkin and Rosell (1962) The muscle dissection was identical to that used for the autoperfused preparation with the exception that the artery to the muscle was cannulated with a blunt needle and perfused with blood from reservoirs Usually two reservoirs were used containing arterial blood at +38° C The blood was stirred constantly with teflon coated magnetic stirrers Both reservoirs were filled simultaneously with arterial blood from a cannulated artery Just as with autoperfusion blood pressure and flow were recorded continuously with a Sanborn direct writing oscillograph

Muscular contractions were induced with bipolar stimulation of the gracilis nerve by means of a Grass S4 impulse generator Stimulation criteria were voltage 1 5 to 3 0 impulse duration 0 1 to 0 4 msec frequency 0 5 to 4 0 impulses per second The muscular contractions produced were single twitches and resulted in moderate shortening since the leg of the animal was not tightly bound to the table The latter was avoided so that no interference of blood flow would result from excessive tension on the muscle The force of contraction was not measured but to get an estimate of the work level oxygen uptake was measured (blood flow × arteriovenous oxygen difference)

The procedure reported in Table I was consistent for each of the 18 animals After completion of the necessary surgery and confirmation of vascular isolation the muscle was covered with mineral oil and the heat lamp turned on 30—60 min were allowed to pass before samples

were collected The 3 samples of muscle were obtained for glycogen analysis, following which 3 paired samples of arterial and venous blood were collected simultaneously at 10 min intervals for glucose and lactate determinations During this time paired samples of blood were obtained for O_2 and CO_2 content Stimulating electrodes were then placed on the nerve to the gracilis muscle and contraction was initiated During the 60 min contraction period paired samples of blood for glucose and lactate measurements were obtained at 5, 10 20, 30, 50 and 60 min Blood for O_2 and CO_2 contents were obtained at 10 and 40 min into the contraction period Upon cessation of stimulation 3 muscle samples were obtained for glycogen analysis No further samples were taken following cessation of stimulation since recovery phenomena were not studied The entire muscle was removed and weighed (range 30 to 66 g) No detectable damage or gross edema was found

Glucose and oxygen uptake were calculated using the Fick principle The % $\dot{V}_{O_2}$ (glucose) shown in Table II was calculated using the following formula

$$\% \dot{V}_{O_2} (glu) = (glu \times 6) / (\dot{V}_{O_2}) \times 100$$

where % $\dot{V}_{O_2}$ (glu) = per cent of total oxygen consumption accounted for by glucose oxidation glu = millimoles of glucose uptake/g tissue 6 = constant (6 mmoles O_2 needed to oxidize 1 mmole glucose) and $\dot{V}_{O_2}$ = total O_2 uptake/g tissue It is understood that this calculation can be made only by assuming that all of the glucose taken up by the muscle was completely oxidized

Chemical Determinations

Plasma glucose was measured enzymatically with the glucose oxidase technique using Gluocostat® (Worthington Biochemical Corp Freehold New Jersey) With constant flow perfusion the glucose concentration in reservoir blood was measured at the beginning and end of each experiment to determine whether any change in concentration had occurred Because the duration of these studies was short little to no change in glucose concentration of arterial (reservoir) blood was found In the autoperfused studies paired arterial and venous blood samples were collected simultaneously and the plasma concentration of glucose analyzed This was done simply by centrifuging the blood removing an appropriate amount of plasma and determining the glucose concentration

In the experiments listed in Table I even greater care was taken in the determination of blood glucose Paired samples of arterial and venous blood were collected and immediately a Somogyi filtrate was prepared At the end of the experiment the glucose concentration was measured with the glucose oxidase technique Each sample was examined in duplicate and read separately by separate technicians Five to ten repeated analyses of one blood sample a study done in three different days showed the standard deviation did not exceed ±1 0 mg/100 ml

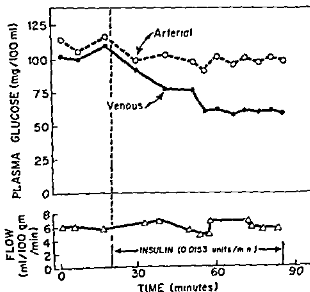


Fig 1 Effect of insulin on a-v glucose difference across isolated resting gracilis muscle Plasma glucose (O —— O arterial ● —— ● venous) concentrations and blood flow (△ —— △) are shown The duration of crystalline zinc insulin infusion (0 0153 units/min) is shown by the arrows

with glucose concentrations in the range of 80 to 100 mg/100 ml blood This was the range of glucose concentration in which the majority of the experiments was performed

Muscle glycogen was determined with the method of Good Kramer and Somogyi (1933) Three samples of muscle each weighing between 0 5 and 1 0 g were taken from different sample *sites immediately before each experiment began and immediately following one hour of muscle* contraction at a frequency of 4 imp/sec Each sample of muscle was dropped immediately into [illegible] the muscle sample [illegible]

[illegible] p glucose from the blood insulin was infused i a. in 4 hr expts (0 015 units/min) The average control glucose uptake was 2 8 μg/g×min and increased to 10 8 μg/g×min following close arterial injection of insulin or addition of insulin to the blood reservoir Fig 1 shows the results from one representative experiment As noted, flow remained relatively constant while the A V glucose difference widened considerably

Results

Glucose Studies in free flow Preparations

Table I summarizes the data from these studies The mean control arterial blood glucose concentration in the three groups of animals was 86, 63 and 72 mg % During the contraction period of 1 hr the mean arterial glucose concentration increased to 93 79 and 82 mg %, respectively Analysis of each individual series of experiments failed to show an influence on glucose uptake with this range of different arterial glucose concentrations

Muscular contraction at 2 imp/sec for 1 hr increased glucose uptake from 0 55 ± 1 63 to 15 33 ± 4 77 μg/g×min, at a contraction frequency of 4 imp/sec, glucose uptake increased from 2 00 ± 0 42 to 23 68 ± 2 34 μg/g×min This same frequency and duration of contraction following the i v injection of propranolol (4 mg/kg) produced almost identical changes in glucose uptake i e, from 2 10 ± 0 44 to 25 80 ± 3 27 μg/g×min At contraction frequencies of 2/sec glucose uptake ranged from 4 2 to 38 1 μg/g×min, 16 0 to 33 8 μg/g×min at 4 imp/sec before propranolol and 13 7 to 38 9 μg/g×min following propranolol

Glycogen Studies Multiple biopsies of muscle were obtained for glycogen analysis (see Methods) before and immediately following one hour of muscular contraction at an impulse frequency of 4/sec These experiments were performed without and with beta adrenergic blockade by i v administered propranolol (4 mg/kg) The mean glycogen content in resting muscles was 0 97 ± 0 18 (range 0 52 to 1 79) and 1 05 ± 0 08 g/100 g muscle (range 0 81 to 1 29) respectively Following 1 hr of contraction the mean glycogen content decreased to 0 26 ± 0 08 g/100 g muscle (range 0 05 to 0 64) in the non beta blocked muscles However in the beta adrenergically blocked muscles less reduction was found in glycogen content following contraction i e 0 56 + 0 07 g/100 g muscle (range 0 37 to 0 73)

Oxygen Uptake The resting oxygen uptake in the 3 groups of muscles was around 0 25 ml/100 g×min With contraction at 2 imp/sec mean oxygen uptake was 3 91 ± 0 40 ml/100 g×min (range 2 60 to 5 17) while contraction at 4 imp/sec increased

TABLE I

	Glucose		Glycogen g/100 g	Lactate a—v diff mg %	Oxygen		
	a—v diff mg %	Uptake µg/g/min			arterial content vol %	venous content vol %	a—v diff vol %
Muscle contraction (2 imp/sec)							
Control	1 4	0 55		—0 52	15 60	10 90	4 70
	±1 7	±1 63		±0 30	±0 87	±1 52	±1 77
Contraction	4 1	15 33		—0 76	16 58	5 95	10 63
60 min	±1 2	±4 77		±0 16	±0 74	±1 08	±1 25
Muscle contraction (4 imp/sec)							
Control	2 7	2 00	0 97	—0 70	18 92	15 08	3 83
	±0 7	±0 42	±0 18	±1 82	±0 72	±1 15	±0 65
Contraction	3 8	23 68	0 26	—1 97	19 95	3 97	15 98
60 min	±0 3	±2 34	±0 08	±1 49	±0 52	±0 39	±0 67
Muscle contraction (4 imp/sec) post propranolol (4 mg/kg)							
Control	3 5	2 10	1 05	—0 82	16 33	12 29	4 04
	±0 7	±0 44	±0 08	±1 17	±0 85	±0 60	±0 66
Contraction	5 5	25 80	0 56	—0 97	17 14	1 77	15 37
60 min	±0 7	±3 27	±0 07	±1 29	±0 85	±0 31	±0 76

mean oxygen uptake to 10 33 ± 0 49 ml/100 g × min (range 9 42 to 11 85) The administration of propranolol reduced oxygen uptake at 4/sec contraction frequency The mean oxygen uptake following propranolol was 7 73 ± 1 00 ml/100 g × min (range 5 87 to 12 8) Of note oxygen extraction from the blood was enhanced in the propranolol blocked muscles as manifested by oxygen A V differences and lower venous blood oxygen contents Since blood flow decreased as well the increased oxygen extraction failed to increase oxygen uptake

Table II contains the same data as some of those shown in Table I but shows in addition mean changes in 4 of the measurements during the first and second 30 min contraction periods At contraction frequencies of 2 imp/sec there was no substantial difference in any of the measurements when the results between the first and second 30 min contraction periods are compared Using contraction frequencies of 4/sec oxygen uptake and muscle blood flow were lower during the last 30 min contraction period as compared to the results obtained during the first 30 min of contraction After administration of propranolol this same frequency of contraction (4 imp/sec) produced different results only in blood flow These data show that while blood flow is reduced during the second 30 min contraction period with both studies using 4 imp/sec (i e 4/sec without and following propranolol) glucose uptake was not changed The last column of Table II shows the percentage of oxygen uptake that can be accounted for by oxidation of glucose taken up from the blood if it is assumed that this glucose is oxidized and not converted for example to glycogen

Carbon dioxide				Arterial Pressure mm Hg	Muscle Blood Flow ml/100 g/min	Hemoglobin g/100 ml
Oxygen uptake ml/100 g/min	arterial content vol %	venous content vol %	a—v diff vol %			
0 28	49 80	51 03	— 1 23	126	12 4	14 1
±0 08	±2 19	±2 56	± 0 47	±4	±3 1	±0 7
3 91	45 92	56 53	—10 62	112	37 5	14 9
±0 40	±2 05	±2 21	± 1 20	±8	±2 6	±0 8
0 27	47 30	48 58	— 1 32	137	8 4	14 9
±0 03	±1 54	±1 73	± 0 48	±4	±1 5	±0 7
10 33	43 85	58 72	—14 87	124	61 9	15 5
±0 49	±1 51	±1 75	± 0 35	±5	±3 7	±0 6
0 23	44 63	46 31	— 1 69	123	6 1	14 7
±0 01	±1 11	±0 72	± 0 64	±4	±0 4	±0 5
7 73	41 40	56 59	—15 19	119	48 5	15 1
±1 00	±0 88	±0 93	± 0 41	±4	±4 4	±0 6

Adrenalin and Glucose Uptake In 5 animals (8 expts) the effect of i v infused adrenalin on the glucose a v difference was investigated The periods of infusion ranged from 9 to 15 min, adrenalin delivery per minute varied from 0 21 to 9 55 μg/min Prior to adrenalin infusion, control glucose uptake averaged 0 70 μg/g× min (range —0 91 to ±5 00), while during the infusion of adrenalin this increased to a mean of 7 70 μg/g×min (range 1 76 % to 23 00) The greatest glucose uptake

Table II

	Glucose uptake μg/g/min	Oxygen uptake ml/100 g/min	Muscle blood ml 100 g/min	% VO₂ (glucose)
Muscle Contraction (2 imp /sec)				
Control	0 55±1 63	0 28+0 08	12 4±3 1	14 7 %
1st 30 min	17 73±5 30	4 10±0 40	39 7+3 0	32 4 %
2nd 30 min	13 03±4 38	3 72+0 39	35 4+2 3	26 3 %
Muscle contraction (4 imp sec)				
Control	2 00±0 42	0 27±0 03	8 4±1 5	55 5 %
1st 30 min	23 68±3 94	10 99±0 81	67 2±5 3	12 0 %
2nd 30 min	22 85±4 10	9 40±0 44	56 7±3 3	18 2 %
Muscle Contraction (4 imp/sec) post—Propranolol (4 mg/kg)				
Control	2 10±0 44	0 23±0 01	6 1+0 4	68 5 %
1st 30 min	23 51±4 07	8 16±1 21	53 1±4 8	21 6 %
2nd 30 min	27 43±3 60	7 29±0 92	44 1+4 1	28 2 %

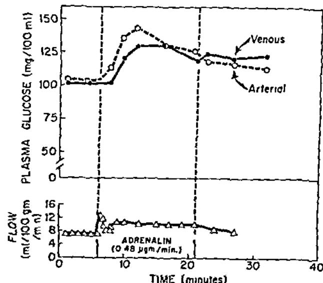


Fig 2 Effect of iv infused adrenalin on blood flow and A V glucose difference across isolated autoperfused gracilis muscle Adrenalin infusion indicated by arrows

occurred with the highest infusion rates of adrenalin which in turn produced the greatest increase in glucose concentration of arterial blood In every experiment glucose uptake was usually maximal during the first 5 min of adrenalin infusion Thereafter the a v difference narrowed until either no glucose uptake occurred or as in one instance a slight loss of glucose from the muscle occurred Fig 2 represents the results obtained from 1 expt As shown, arterial plasma glucose concentration increased and while the glucose concentration in venous blood draining the muscle also increased the values obtained were lower than those measured in the arterial blood This increase in a v difference and blood flow indicates an uptake of glucose by the muscle

Comments on These Data

The skeletal muscle preparation used responds to injected insulin with an increased uptake of glucose It can also be concluded that a substantial uptake of glucose was *demonstrated over the isolated gracilis muscle during contraction* The RQ—determined on exercise arterial and venous blood samples—was around or above 0 95 indicating that approximately 80 per cent of the substrate for aerobic energy delivery came from carbohydrates During contraction 10—30 per cent of this could be accounted for by the observed glucose uptake Our glucose uptake and its relative role for the total energy output is however far less than reported by Chapler and Stains by (1968)

From the above presented data it is impossible to come to a conclusion whether it is the muscle contraction per se that augment the glucose uptake or some other factors Goldstein (1961) for example has proposed that during exercise a substance is produced which enhances the uptake of glucose in the muscle In the free-flow

TABLE III

Dura tion Stim	Stim Fre quency	Control Flow	Stim Flow	Control Glucose a—v diff	Stim Glucose a—v d ff	Control Glucose Uptake	Stim Glucose Uptake	Control Oxygen Uptake	Stim Oxygen Uptake	
10 5	0 5	5 3	6 8	7 0	0 0	2 49	0 00			
14 0	0 5	6 6	7 7	0 0	0 0	0 00	0 00			
8 0	1 0	7 1	8 0	2 0	0 0	0 98	0 00			
4 0	1 0	6 3	6 3	4 5	3 5	1 81	1 41			
15 0	0 75	7 7	17 2	8 5	4 0	4 71	0 42	0 42	1 85	—
15 0	0 75	4 3	14 0	7 7	3 1	2 58	4 38			—
16 0	1 0	4 8	9 5	5 0	0 0	1 58	0 00			—
13 0	1 0	7 1	9 7	6 5	1 0	3 14	0 66			
31 0	2 0	11 2	19 0	5 5	0 5	4 19	0 64			—
12 0	1 0	10 0	11 3	23 0	22 0	13 80	14 92			*

Table showing effects of *muscular contraction using reservoir blood for perfusion of isolated gracilis* muscle Duration stim (min) stim frequency (imp sec), flow (ml/100 g/min), glucose a—v diff (mg/100 ml plasma, glucose uptake (μg/g/min) Oxygen uptake (ml/100 g/min)

Constant flow perfusion
+ Flow from reservoir increased during contraction
* Perfusion with blood containing insulin (0 2 units ml)

experiments the blood recirculated in the animal throughout the study Goldstein's exercise factor can therefore not be excluded Further, as arterial blood did come from the systemic arterial flow, the blood concentration of other substances such as hormones may have been changed during the experiment In order to exclude these possibilities another set of experiments was performed where the blood perfusing the gracilis muscle was taken from a reservoir and all venous blood discharged

Constant Flow Preparation (blood from reservoir)

Muscular Contraction and Glucose Uptake Table III shows the results obtained from 10 expts in 8 animals During the contraction period there was no widening of the glucose a v difference as compared to resting control values Instead there was a tendency for the a v difference for glucose to narrow even though blood flow was held constant or slightly increased during the contraction period The last experiment shown in Table III was also performed with constant flow perfusion but the reservoir blood contained insulin (0 2 units/ml) In this experiment muscle contraction failed to augment further the increased glucose uptake by insulin *i e* the combination of insulin and muscular contraction did not result in an uptake of glucose greater than that measured with insulin alone Fig 3 shows the results from 1 expt and is representative of the constant flow series

Effect of adrenalin In 4 animals (8 infusions) adrenalin was infused In none of these muscles did adrenalin produce an uptake of glucose greater than that present prior to adrenalin infusion In each instance the concentration of adrenalin used was sufficient to produce vasoconstriction as evidenced by an increase in perfusion pres-

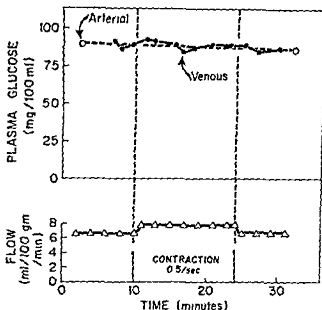


Fig 3 Effect of muscular contraction on a v glucose difference across isolated constant flow perfused gracilis muscle Symbols as in Fig 1 Contraction period enclosed in dashed lines

sure in the presence of constant blood flow Fig 4 shows the results from a representative experiment

Experiments were also done in which during constant flow perfusion, adrenalin infusion (close arterial) and muscular contraction were combined The combination of low frequencies of contraction used in these experiments and adrenalin infusion failed to demonstrate any alteration in glucose uptake by the muscle Fig 5 shows the results from one study

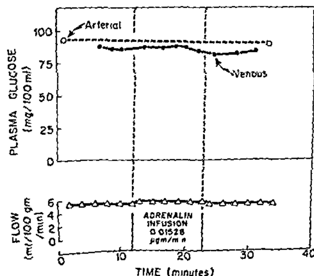


Fig 4 Effect of close arterial infusion of adrenaline on a v glucose difference across isolated constant flow (reservoir) perfused gracilis muscle

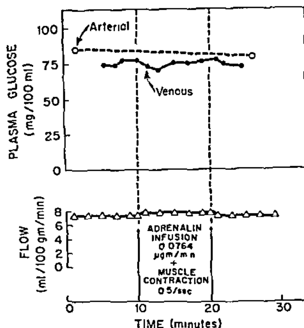


Fig 5 Effect of combining close arterial infusion of adrenaline and muscle contraction on a v glucose difference across isolated constant flow (reservoir) perfused gracilis muscle

Effect of Abruptly Changing the Arterial Glucose Concentration In view of the correlation between increased arterial glucose concentrations induced by iv adrenalin infusions and glucose uptake by the isolated gracilis muscle 6 expts (4 animals) were done to investigate the effect of abrupt changes in arterial blood glucose concentration on uptake of glucose by skeletal muscle Two blood reservoirs were used and were filled simultaneously from the same donor Glucose was added to one reservoir of blood to increase the concentration of glucose to approximately 250 mg/100 ml plasma By suddenly changing from one reservoir to the other it was possible to abruptly increase the concentration of glucose in the blood perfusing the muscle and make frequent measurements of venous glucose concentrations when blood flow remained constant Fig 6 shows that a substantial glucose uptake resulted with this procedure since several minutes were required for the venous glucose concentration to reach a new equilibrium Similarly abruptly returning the arterial blood glucose concentration to normal resulted in a transient net loss of glucose from the muscle tissue which would of course include interstitial fluid etc In two additional animals this same procedure was repeated with the exception that muscular contraction was begun before or simultaneously with the change in arterial blood glucose concentration This combination of events did not alter the type of response shown in Fig 5 Finally in one additional animal the arterial glucose concentration was lowered abruptly from a normal of 97 to 63 mg/100 ml plasma The venous glucose concentration rapidly approached but did not fall below the arterial level On return to a normal arterial concentration of glucose the venous concentrations lagged briefly with the result that transient shifts in glucose a v difference occurred

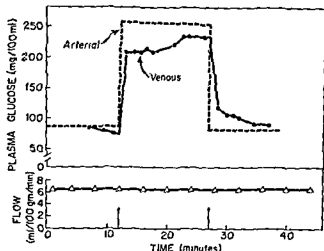


Fig 6 Effect of abrupt increase in arterial (reservoir) blood glucose concentration on a v glucose difference across isolated, constant flow perfused gracilis muscle

Discussion

There is a striking difference in glucose uptake during contraction comparing experiments with the muscle perfused from the systemic circulation free flow and from a reservoir The free flow experiments showed definite increase in glucose and oxygen uptake during the contraction period The uptake of both was considerably more enhanced at the higher frequency of contraction (Table I) Glycogen content was also markedly reduced in muscle samples taken immediately after cessation of the 1 hr contraction period Further, the output of lactate from the muscles during contraction was greater at higher frequencies of contraction While not calculated it is clear from the data in Table I that the metabolic RQ was quite high ($\geqslant 0.95$) during muscular contraction There is little question, then, that in these studies carbohydrate metabolism is enhanced during muscular contraction

The A-V difference for glucose did not widen during contraction over the reservoir perfused muscle It should be noted that this type of preparation facilitates the possibilities to detect a change in a v difference as blood flow changes are minimal during the stimulation period On the other hand, the lack of increase in blood flow during contraction limits the increase in energy output It is however increased, and no oxygen is found in the blood leaving the muscle during and between contraction lactate is also increased Thus also in this set of experiments carbohydrate metabolism seems to be enhanced, but muscular contraction does not affect the glucose uptake Part of the difference in results between the two types of experiments may be the difference in energy output but also other factors must be of importance Most likely the recirculation of blood from the exercising muscle results in the release of substances that facilitate the uptake of glucose in the muscle From the results in the present study Goldstein s (1961) exercise factor cannot be excluded either

Comparing our data (systemic flow perfusion) with those obtained in a similar study by Chapler and Stainsby (1968), several striking similarities and differences were observed While each study employed *in situ* dog skeletal muscles the gracilis muscle was used in the present study and the gastrocnemius plantaris muscle was used in that by Chapler and Stainsby In the gastrocnemius plantaris muscles glucose uptake during resting control conditions was 5 times that seen in gracilis muscles Resting oxygen uptake was also approximately 4 times greater in the gastrocnemius than in the gracilis muscle During contraction at 5 imp/sec, glucose uptake increased to an approximate average of 80 μg/g×min in the gastrocnemius but only 25 μg/g×min in gracilis muscles contracted at 4 imp/sec Oxygen uptakes however were quite comparable in the two studies being about 14 ml/100 g×min in the gastrocnemius (5 imp/sec) and 10 ml/100 g×min in the gracilis (4 imp/sec Thus with comparable oxygen uptakes in the two studies with comparable frequencies of contraction glucose uptake in the gracilis muscle was less than 30 % of that seen in the gastrocnemius plantaris muscle There does not appear to be any ready explanation for these different results as they are both mixed muscles as regards red and white fibers (Edstrom and Nystrom 1969)

An attempt was made to ascertain whether or not glucose uptake was related to net glycogen breakdown *i e*, if more glycogen was available for metabolism would this result in less glucose uptake? In the present study beta adrenergic block with propranolol resulted in less net glycogen breakdown with a given frequency of contraction and as well a substantial reduction in oxygen uptake, glucose uptake however was the same with or without propranolol Propranolol is a pharmacological agent and produces many metabolic changes (Maling *et al* 1966 a b Master and Glaviano 1969), so that it would be difficult to attempt to answer the above question on the basis of these data On the other hand Chapler and Stainsby (1968) found less glycogen breakdown than measured in the present study and as well a greater uptake of glucose These authors found that only 36 % of the glycogen was broken down with contraction at 5 imp/sec for 60 min while in the current study which employed contraction frequencies of 4 imp/sec for the same time period approximately 73 % of the glycogen was broken down The latter compares favorably with glycogen studies in humans (Hermansen Hultman and Saltin 1967) The data relative to the question do not appear adequate to either support or refute the possibility that glycogen availability influences the amount of glucose taken up by contracting muscle However the data are sufficiently suggestive to warrant consideration of the possibility

Data from Table II which illustrate the changes in several measurements during the first and second 30 min contraction periods both without and with beta adrenergic blockade help to answer some additional questions For example muscle contraction at 4 imp/sec produced substantial blood flow differences in the first and second 30 min contraction periods however this change in blood flow did not change glucose uptake in the two 30 min periods but did alter oxygen uptake Following propranolol blood flow decreased significantly during the second 30 min of

contraction, but no change in either glucose or oxygen uptake occurred These data would seem to have pertinence to the comparison of the described changes in gracilis and gastrocnemius muscles Blood flow increases to a greater extent in gastrocnemius muscle during contraction, hence it could be postulated that the striking differences in glucose uptake in the two muscles would relate to greater blood flow in gastrocnemius muscle The data in Table II would tend not to support such a view

Our studies do not include any studies on fat metabolism in working muscle and cannot evaluate the interrelationship between fat and carbohydrate metabolism of skeletal muscle during contraction Data from other studies have shown clearly that when glucose oxidation is normal, impaired or facilitated free fatty acids always accounted for the major portion of external energy supply to working muscles (Paul, Issekutz and Miller 1966, Issekutz, Paul and Miller 1967) These quoted studies indicate that no more than 10—15 % of the energy expended by muscular contraction is supplied from plasma glucose, the remainder of the extramuscular fuel being derived from fat metabolism These studies obviously imply that some mechanism is available to limit glucose uptake by contracting skeletal muscle, especially at high frequencies of contraction when energy expenditure is the greatest

As shown in Table II muscular contraction at 2 imp/sec produced an uptake of glucose which, if completely oxidized would account for 29 % of the oxygen uptake Muscular contraction at 4 imp/sec produced greater uptakes of glucose but in contrast to the above could account for no more than 17 % of the oxygen uptake These calculations are based on the assumption that all of the glucose taken up from blood is completely oxidized Since it was not possible to determine how much of the glucose taken up was oxidized and how much was diverted for example to form glycogen no definitive statement can be made relative to this finding

In view of the limited extramuscular pool of carbohydrates (liver and extracellular fluid) which probably amounts to 40—80 grams in humans and the inability of glucose 6 phosphate to leave the muscle cell it seems logical that muscle with its enormous ability to increase metabolism could not depend on exogenous glucose to meet metabolic demands Thus the increased glucose utilization found during heavy muscular work in humans or animal preparations—intact awake animals or isolated limbs—would be more related to metabolism of nervous tissues of fat tissues that are dependent upon glucose as their energy source and have little or no glycogen stored The present results with a relative lack of glucose uptake by contracting skeletal muscle would preserve the limited extramuscular glucose pool allow blood glucose to be maintained at physiological levels and maintain the integrity of the organism by keeping this fuel available to those vital tissues that utilize only or primarily glucose

The authors wish to express their appreciation to Mr James A Hedgman Mr Julius Lamar Mr David Rogers, and Miss Janet Parks for their expert technical assistance

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Acta physiol scand 1971, 81 138—140
From the Department of Physiology, Royal Veterinary College Stockholm Sweden

Is the Adaptation of the Muscle Spindle of Ionic Origin?

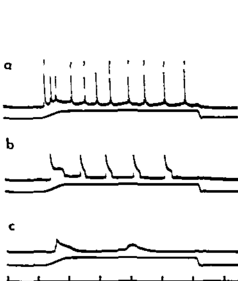
By

I Husmark and D Ottoson

The receptor potential of the muscle spindle evoked by a steplike stretch consists of an initial transient phase followed by a decline to a more or less steady level The characteristic decline of the response from the dynamic peak to the static level is generally attributed to the viscous properties of the intrafusal muscle fibres (*cf* Houk Cornew and Stark 1966, Toyama 1966) Recent photomicroscopical studies on the length changes of frog spindles suggest, however, that the intracapsular portions of the intrafusal fibres behave as if they were almost purely elastic (Ottoson and Shepherd 1968 1970) There is also evidence from experiments on frog spindles subjected to constant load (Husmark and Ottoson 1971) that the adaptive decline of the response is only partly to be attributed to relative length changes within the spindle This raises the question as to the origin of the non mechanical component of adaptation One possibility is that the decline of the response is related to the ionic processes underlying the transducer action

It has been established that potassium ions play an important role in the repolarization process of nerve fibres Since the adaptive decline of the receptor potential may be regarded as a partial repolarization of the sensory endings it appeared conceivable that potassium ions might be involved also in this process The present study was carried out to test this hypothesis The experiments were performed on isolated frog spindles This preparation has the advantage that the ionic environment of the spindle can easily be controlled Furthermore by recording from the afferent fibre at its issue from the capsule the effect of ionic changes on the conducted activity as well as on the transducer action can be studied

It was found that removal of potassium from the external solution caused an increase of the spontaneous firing of the spindle and a progressive lengthening of the impulses which developed into heart like action potentials With prolonged soaking (*1—2 hours*) the spontaneous activity decreased and the action potentials became gradually smaller until they finally disappeared These changes were accompanied by a gradual reduction in the responsiveness of the spindle to stretch An example of this is shown in Fig 1 Record *a* shows the response to a stretch of 200 msec duration when the spindle was kept in normal Ringer In *b* is seen the response to the same stretch about 35 min after that potassium had been removed from the bathing solution The response consists of a low frequency discharge of heart like action po



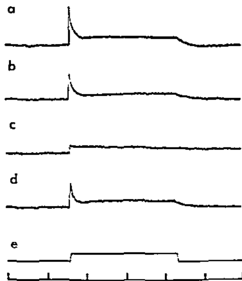


Fig 1   Fig 2

Fig 1 Effect of removal of potassium on response of spindle to stretch *a*, response in normal Ringer, *b*, 35 min *c*, 50 min after removal of potassium from bathing solution Time marks 50 msec

Fig 2 Effect of removal of potassium on receptor potential of muscle spindle *a* response in normal Ringer to steplike stretch indicated in *e* *b* 60 min, *c* 200 min after removal of potassium from bathing solution, *d*, revocery 60 min after return to normal Ringer Time marks 50 msec

tentials About 15 min later there remained only small wave like elevations (record *c*)

To study the effect on the receptor potential experiments were carried out on spindles treated with tetrodotoxin to block the impulse activity The records in Fig 2 illustrate the main results obtained The spindle was stretched with a steplike stretch The response in normal Ringer is shown by record *a* After the spindle had been kept in potassium free solution for about 60 min there was a marked reduction in height of the dynamic peak (record *b*) The static phase was also reduced although to a less extent The dynamic overshoot became gradually smaller with prolonged soaking and was almost abolished after about 200 min (record *c*) It may be noticed that at this stage there was still a static response of appreciable amplitude Together with the reduction of the dynamic peak there was also a gradual prolongation of the fall of the response after release of stretch At the time when the dynamic overshoot was almost abolished the decline of the potential to baseline following termination of stretch lasted several hundred msec

If sodium ions are responsible for carrying the main part of the current of the receptor potential the most plausible explanation of the observed effects appears to be that they are caused by a delayed sodium inactivation corresponding to that respon-

sible for the production of prolonged action potentials in the giant axon (*cf* Baker Hodgkin and Meves 1964) In addition to this a reduction of the resting membrane potential must be postulated since not only does the dynamic overshoot disappear but there is also a marked decrease in height of the static phase of the response

The decrease and final disappearance of the dynamic overshoot in potassium free Ringer has interesting implications as to the interpretation of the mechanism for adaptation of the spindle *As mentioned above there is experimental evidence that the adaptation in terms of the fall of the spindle response is only partly of mechanical origin* The present findings suggest that the remaining component is related to the ionic processes involved in the transducer action of the endings

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From the Institute of Neurophysiology University of Copenhagen, Denmark

A Method to Fill Glass Microelectrodes by Local Heating

By

Thomas Zeuthen

Tip potentials of glass microelectrodes and increased conductivity near the tip have been attributed to hydration of the glass (Agin 1969) Since this hydration is a function of time and temperature (Holland 1964) it should be reduced if the pipette is filled directly by some method which does not involve soaking the electrodes in a solution of electrolyte for hours The method using glass fibers has not been satisfactory in our hands since we have not been able consistently to achieve the low impedances reported by Tasaki *et al* (1968) I have, therefore, developed another method using heat to condense fluid within the tip My method has the advantage that the tips of the electrodes are not damaged mechanically or by crystallization Nonetheless the tip potentials are the same as when the electrodes are filled by the conventional methods of boiling and suction, or boiling in alcohol (Tasaki 1954 Agin and Holzmann 1966) and twice as great as those obtained by Lassen and Sten Knudsen (1968) with another method of filling without boiling

The shaft of the pipette (Jena G 20 with inner and outer diameters of 1 2 and 1 8 mm) is filled with distilled water through an hypodermic needle The pipette is placed in a spiral of constantan wire (Fig 1 a, length 19 mm diameter of the wide end 3 mm of the narrow end 1 5 mm 16 turns, thread diameter 0 5 mm impedance 1 Ω) The wire is heated at a current of 3 amp for 10 sec and then by a current of 1 amp (Fig 1 b) to bring the aqueous vapour in the pipette to equilibrium with the fluid in the shaft The pipette is then advanced in the coil so that the water condenses in the tip and the enclosed air is forced into the shaft of the electrode If the electrode is advanced too fast bubbles form in the shank but can be removed by withdrawing the electrode and then advancing it again The current is switched off when the electrode cannot be advanced further through the narrowing end of the spiral (Fig 1 c)

We use a small motor to advance the electrode at a rate of 1 mm/min for electrodes having shanks 15 mm long 10 mm/min if the shanks are 5 mm long and we watch the pipette through a dissection microscope with an amplification of 10× A bubble of air in the shoulder (Fig 1 d) is washed out when water is replaced by the desired solution of electrolyte injected into the shaft by a hypodermic needle Water in the shank is largely replaced by injecting the electrolyte solution through a glass cannula (50 μ in diameter) advanced to one mm from the tip The remaining water

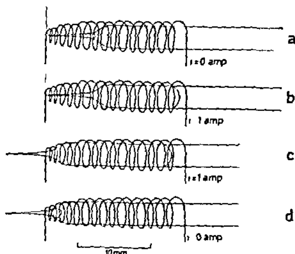


Fig 1 The filling procedure as described in the text

in the tip is replaced by immersing the tip of the electrode in the solution of electrolyte for 20 min just before use

The two electrode tips obtained by pulling the glass in a horizontal puller (Alexander and Nastuk 1953) are identical in shape as seen in a scanning electron microscope With a given electrolyte solution the impedance was calculated from the shape of the electrode (Amatniek 1958), and the impedance of its mate was measured The calculated and measured impedances were identical within 40 %, which corresponds to the uncertainty of 20 % in determining the dimensions

Electrodes filled with 2 5 M KCl have impedances of 5—60 MΩ in Ringer's solution (115 mM NaCl 2 mM KCl 1 8 mM $CaCl_2$ 2 5 mM phosphate buffer pH 7 3) and those pulled under the same conditions have the same impedance to within 10 % The method is adaptable to double barrelled electrodes and to glass capillary tubes with closed tips

Dr Guld and Dr Engbæk initiated this study in the course of their investigation of leakage in microelectrodes The study was supported by the Danish Government Fund for Scientific and Industrial Research

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From the Department of Clinical Pharmacology, Karolinska Hospital, Stockholm

Ontogenetic Development of Cholinergic Receptor Function in Guinea Pig Ileum

By

L O Boréus and D M McMurphy*

It has been shown that cholinergic receptor function is well developed in tissues from human fetuses early from the beginning of the second trimester of pregnancy (Boreus 1967, 1968) Analyses of the dose effect curves for the spasmogenic action of acetyl choline on ileum disclosed that its affinity for the cholinergic receptor, as judged from the pD_2 values, did not change during the development of the fetus in the gestational age period of 12 to 24 weeks Thus, the concentration of acetylcholine needed for 50 % response did not change in spite of the fact that the intensity of the response itself rapidly increased with fetal age

In order to find out whether a similar uniformity in the drug concentration-response relationship exists also at other stages of ontogenetic development, the cholinergic receptor function in ileum of fetal, newborn, young and adult guinea pigs has been analyzed in this report by comparing acetylcholine dose effect curves obtained from *in vitro* experiments in the various ages

At least two consecutive and cumulative dose effect curves for acetylcholine (dose range 0 005—50 μg/ml) were determined on 1 to 2 cm ileal segments from 33 guinea pigs of various ages (Table I)

One or two ileal segments were mounted in a jacket-warmed 20 ml overflow bath with Tyrode solution at a constant temperature of 37° C and bubbled continuously with a gas mixture of 93 5 vol % O_2 and 6 5 vol-% CO_2 Acetylcholine iodide (0 1—0 3 ml) was added directly to the bath and the resulting isometric tension was measured with strain gauge transducers and recorded on a polygraph Details of the method have been described earlier (Boréus 1967)

From the dose effect curves, determinations were made for each animal of the mean acetylcholine dose which gave 50 % of the maximum contractile response

Table I Age and weight of guinea pigs

Age (days)	Number	Wt (g)	Ave
Fetuses last week of pregnancy	7	15—67	42
0—7	9	80—147	102
8—14	3	160—163	162
15—21	5	165—292	210
22—33	6	227—366	290
Adult	3	750—851	833

* Trainee of US Public Health Service Training Grant 5 TO1 HD 00166 03

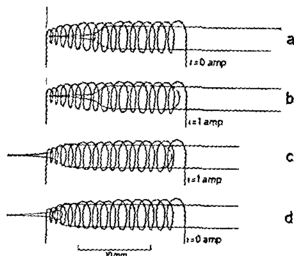


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From the Department of Pharmacology, School of Medicine, Linkoping Sweden

Cyclic AMP as a Mediator of Hormonal Metabolic Effects in Brown Adipose Tissue

By

ANDRAS BEVIZ, LENNART LUNDHOLM and ELLA MOHME LUNDHOLM

Received 15 May 1970

Abstract

BEVIZ, A , L LUNDHOLM and E MOHME-LUNDHOLM *Cyclic AMP as a mediator of hormonal metabolic effects in brown adipose tissue* Acta physiol scand 1971 *81* 145—156

The role of c cl c AMP fo [illegible]

tryptamine also reduced the glucose 6 phosphate content Noradrenaline increased both the total and the phosphorylase *a* activity Addition of cyclic AMP ($4\,5 \times 10^{-6}$ moles/ml) to the suspen [illegible]

O_2 consumption, the glycerol and lactate productions and the FFA release of brown adipose tissue Theophylline potentiated the effect of cyclic AMP on the O_2 consumption and in combination the drugs reduced the ATP and glucose 6 phosphate content of the tissue and increased the phosphorylase activity The magnitude of the metabolic effects was equivalent to the maximal effect of noradrenaline The increase of AMP produced by cyclic AMP was blocked by theophylline It is suggested that the studied metabolic effects of noradrenaline is mediated by an increased formation of cyclic AMP in brown adipose tissue

The calorigenic action of the catecholamines has a complex background It is probable, however that an increased formation of cyclic AMP in different tissues is of importance for this effect (review Lundholm Mohme Lundholm and Svedmyr 1968)

The catecholamines and some other hormones (ACTH glucagon 5 hydroxy tryptamine vasopressin TSH) stimulate the oxygen consumption lipolysis lactate production and phosphorylase activity in white adipose tissue (Ball and Jungas 1965 Steinberg and Vaughan 1965) In brown adipose tissue the stimulating action of these hormones on the oxygen consumption is very marked whereas the lipolytic effect is not so pronounced (Joel 1965) We have in preliminary reports given

evidence that besides these effects noradrenaline reduced the content of energy rich phosphate compounds and hexosephosphates in brown adipose tissue but at the same time increased the phosphorylase activity Some of these effects could be reproduced by addition of cyclic AMP (Beviz and Mohme-Lundholm 1967, Beviz, Lundholm, Mohme Lundholm 1968) The lipolytic effect of the catecholamines, ACTH and glucagon in white adipose tissue are probably mediated by an increased formation of cyclic AMP (Butcher 1966, Butcher *et al* 1968) Reed and Fain (1968) have reported that dibutyryl cyclic AMP stimulates the oxygen consumption of brown adipose tissue In the following we present in more detail the metabolic effects of these hormones in brown adipose tissue and the evidence indicating that cyclic AMP is a mediator of these actions

In seeking support for this hypothesis we studied the following questions

1 Does noradrenaline increase the formation of cyclic AMP in brown adipose tissue in the same concentration as produces the metabolic effects?

2 Have other hormones (ACTH, 5-hydroxytryptamine) which also stimulate cyclic AMP formation in adipose tissue the same metabolic actions as noradrenaline?

3 How are the metabolic actions of noradrenaline and other hormones influenced by an adrenergic β receptor blocking agent?

4 Does theophylline which inhibits the enzymatic hydrolysis of cyclic AMP, produce the same metabolic actions as noradrenaline?

5 Can the metabolic effects of noradrenaline be reproduced by cyclic AMP or any of its derivatives?

Methods

The tests were performed on interscapular brown adipose tissue from rats (Sprague Dawley rats raised by Anticimex) weighing 300—400 g and approximately 6 months of age It was found that there were seasonal variations in the metabolic responses of the brown adipose tissue, which to some degree were dependent on the degree of adaptation to cold of the animals In order to reduce these variations the animals were kept at a temperature of 23° C for 6—10 days before being used There is probably also a variation of the metabolism of the fat tissue dependent on the age of the animals (Ball and Jungas 1965) As we were unable to raise our own animals we had no adequate check of the animals ages By using fully grown animals however we hoped to reduce this cause of variation

Despite this cautiousness there were significant variations in the basal values and hormonal effects between different test series (Table I and II) We have therefore avoided in most cases drawing conclusions from results obtained in different series of tests

The animals (1—4 per test were killed by a blow on the neck and the brown fat tiss e was [illegible] of scissors weighed [illegible] and with the addi [illegible] mption according to Warburg the amount of tissue used was about [illegible] minations f ATP CrP, hexose phosphateesters or cyclic AMP about 200 mg/2 ml was used When the oxygen consumption and production of FFA glycerol and lactate was measured the tissue was incubated for 60 min at 37 C at a O_2 tension of about 700 mm Hg when the content of ATP ADP, AMP, CrP and G 6-P was determined the time of incubation was 30 min One part of the tissue served as a control and another part was incubated with the respective hormones In the tests when an adrenergic β receptor blocking agent was used one part was incubated with this agent and another part with the hormone too

The cyclic AMP content of the tissue was determined according to the method of Breckenridge (1964) modified by Beviz and Lundholm (unpublished) The tissue contents of adenosine

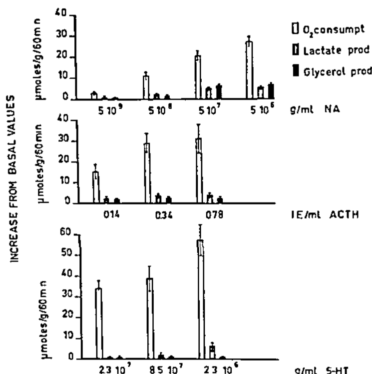


Fig 1 Dose response relationship between the effect of noradrenaline (NA), ACTH and 5-hydroxytryptamine (5 HT) on the oxygen consumption and lactate and glycerol productions of brown adipose tissue from the rat Mean ± S E M of 4—6 tests

triphosphate (ATP) cr [illegible] P) were determined according to ar [illegible] Mohme Lund holm 1964) The conte [illegible] and 1963) and FFA (Ducombe 1964) [illegible] the incubation period The phosphoryl [illegible] *l* (1962) The following drugs were used

L-noradrenaline HCl (Nor Exadrin® Astra) ACTH (Isactid® Ferring) 5 hydroxytryptamine (5 HT) Sigma)

dl 4 (2 isopropylamino-1 hydroxyethyl) methane sulfon anilide-HCl (sotalol MJ 1999 Mead Johnsson Evansville U S A)

cyclic AMP and dibutyryl cyclic AMP (Boehringer Mannheim) albumin (bovine) fatty acid poor (Calbiochem)

Results

Influence of noradrenaline on the cyclic AMP content of brown adipose tissue After 15 min of incubation noradrenaline (5×10^{-6} g/ml) had raised the content of cyclic AMP in the tissue from a mean value of 3 0 ± 0 4 nmoles/g wet weight to 5 1 ± 1 4 nmoles/g This increase of 2 1 ± 0 4 was statistically significant ($P < 0\ 01$)

TABLE I Influence of noradrenaline (5×10^{-6} g/ml) and sotalol ($6\ 8 \times 10^{-6}$ g/ml) and noradrena-
tests with noradrenaline and sotalol separately the change is related to the basal value.
of the blockade is calculated from the difference noradrenaline change — sotalol + nora-
$P < 0\ 01$, *** = $P < 0\ 001$ Mean ± S E M of 7 tests

	O_2 consumpt µmoles/g/60 min	Production of (µmoles/g/60 min)		
		lactate	glycerol	FFA
Basal values	25 3 ± 1 8	7 3 ± 1 3	2 1 ± 0 4	8 0 ± 0 5
Noradr change	17 6 ± 1 7***	1 0 ± 0 2**	2 9 ± 0 4***	3 9 ± 0 9**
Sotalol change	0 6 ± 1 6	0 5 ± 0 6	0 3 ± 0 6	0 1 ± 0 7
Noradr + Sotalol change from Sotalol values	4 8 ± 1 5*	—0 3 ± 0 5	—0 2 ± 0 9	0 2 ± 0 4
Sign of blockade	***	*	*	**

Influence of noradrenaline, 5-hydroxytryptamine (5-HT) and ACTH on the oxygen consumption, glycerol and lactate production, ATP, CrP and G-6-P contents of brown adipose tissue Noradrenaline increased the oxygen consumption and the FFA and glycerol release and reduced the content of high energy phosphate compounds and G 6 P of brown adipose tissue (Fig 1, Table I) It was of interest to study whether other hormones, which according to Butcher, Baird and Sutherland (1968) stimulate the formation of cyclic AMP in fat tissue, had the same metabolic actions as noradrenaline *In Fig 1 the dose-response relationships for some metabolic* actions of noradrenaline, 5-HT and ACTH are shown As can be seen the hormones stimulated to different degrees the oxygen consumption and lactate and glycerol productions The action of 5-HT on the oxygen consumption was especially pronounced In further experiments we studied the metabolic actions of the different

TABLE II Influence of ACTH (0 34 IU/ml), 5 hydroxytryptamine (5-HT) ($2\ 3 \times 10^{-6}$ g/ml) theo- cyclic AMP (0 45 µmole/ml) on the metabolism of brown adipose tissue, and the effect of an action by sotalol is denoted by asterisks, see text to Table I Mean ± S E M

Parameter	Number of tests	O_2 cons	Production of (µmoles/g/60 min)		
			lactate	glycerol	FFA
ACTH basal values	6	27 5 ± 2 0	6 6 ± 0 6	0 7 ± 0 3	—
ACTH change		16 5 2 9***	1 4 ± 0 6*	0 9 ± 0 3*	—
ACTH + sot change		1 1 ± 1 0	1 0 ± 0 5	0 6 ± 0 2*	—
sign of blockade		**	*	N S	
5-HT basal values	7	31 3 ± 2 2	7 6 ± 0 7	0 3 ± 0 05	—
5-HT change		57 4 ± 7 5***	6 3 ± 1 3***	1 0 0 3*	—
5-HT + sot change		0 0 ± 0 4	0 1 ± 0 5	0 0 ± 0 1	—
sign of blockade		***	**	*	—
Dibut -cyclic AMP basal values		23 3 ± 1 3	—	—	6 2 0 2
Dibut change	13	11 7 ± 3 3**			2 2 0 5**
Dibut + sot change	7	9 1 ± 3 2*			—0.2 ± 1 0
sign of blockade		N S			*

line and sotalol in combination on the metabolism of brown adipose tissue from the same rats In In tests with noradrenaline+sotalol the change is related to the sotalol values The significance drenaline change in paired tests, the significance levels are denoted by * = P < 0.05, ** —

Content of (μmoles/g)		
ATP	CrP	G 6 P
0.21 ± 0.03	0.076 ± 0.009	0.048 ± 0.007
—0.077 ± 0.018**	—0.036 ± 0.017*	—0.021 ± 0.017***
—0.055 ± 0.014**	0.022 ± 0.005***	—0.007 ± 0.009
—0.017 ± 0.017	—	—0.008 ± 0.012
*	—	N S

hormones in a concentration which seemed to have had a maximal effect (Table I and II) Beside the mentioned effects all hormones decreased the ATP content Noradrenaline and 5 HT also reduced the CrP and glucose 6-phosphate contents of the tissue There was, however, a quantitative difference between noradrenaline and 5 HT, the calorigenic action of the latter was stronger than that of noradrenaline but its stimulation of glycerol production was weaker

Influence of sotalol on the metabolic actions of noradrenaline, 5 HT and ACTH The adrenergic β receptor blocking agents have been suggested by Sutherland *et al* (1968) to act by inhibiting the formation of cyclic AMP We have found that sotalol in a concentration of 3×10^{-5} g/ml blocked the cyclic AMP stimulating action of adrenaline in isolated rat diaphragm (Beviz and Lundholm 1970) In a preliminary study (Fig 2) we tested out the dose of sotalol needed to block some of the metabolic

phylline (4.5×10^{-4} g/ml) cyclic AMP (4.5 μmoles/ml) theophylline+cyclic AMP and dibutyryl of sotalol (6.8×10^{-6} g/ml) on these actions The significance level of an observed effect or blockade

Content of (μ moles/g)		
ATP	CrP	G 6 P
0.19 ± 0.02	0.13 ± 0.02	0.003 ± 0.01
—0.05 ± 0.02*	0.0 ± 0.01	0.00 ± 0.01
—	—	—
0.23 ± 0.03	0.09 ± 0.01	0.04 ± 0.004
—0.13 ± 0.04*	—0.06 ± 0.02**	—0.01 ± 0.01
—	—	—
—	—	—
—	—	—

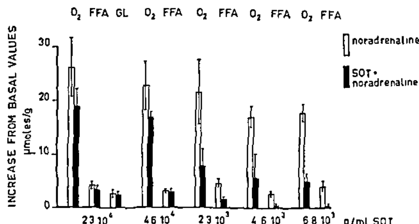


Fig 2 Influence of noradrenaline (5×10^{-6} g/ml) and sotalol + noradrenaline on oxygen consumption (O2) *FFA release (FFA) and glycerol production (GL)* in brown adipose tissue Influence of sotalol in increasing concentrations Mean ± S E M of 4—6 tests

actions of noradrenaline It was found that only above a concentration of $2\,3 \times 10^{-3}$ g/ml did sotalol significantly block the effects of noradrenaline For a more pronounced inhibition of the noradrenaline effect a still higher concentration of sotalol was needed ($6\,8 \times 10^{-3}$ g/ml) It is possible that the low lipid solubility of sotalol may explain its rather weak effect in these tests (Lish *et al* 1967)

In this concentration sotalol itself did not significantly influence the O_2 consumption or the lactate glycerol or FFA production It lowered the ATP content, however but raised the CrP content (Table I) The metabolic effects of noradrenaline were either reduced (O_2 consumption, ATP and G-6 P) or totally blocked (lactate glycerol and FFA release) (Table I)

Sotalol also completely blocked the calorigenic and lactate producing actions of ACTH and 5 HT and the glycerol producing action of 5-HT but not that of ACTH (Table II)

Metabolic actions of theophylline in brown adipose tissue Theophylline inhibits the enzymatic hydrolysis of cyclic AMP (Sutherland *et al* 1968) and it was of

TABLE III Influence of cyclic AMP (4 5 μmoles/ml) and theophylline ($4\,5 \times 10^{-3}$ g/ml) and cyclic ADP and AMP content of brown adiopse tissue Significance of potentiation denotes if

	O_2 consumpt μmoles/g/60 min	Production of (μmoles/g/60 min)		
		lactate	glycerol	FFA
Number of tests	8	8	8	8
Basal values	22 5 ± 1 3	7 7 ± 0 4	0 8 ± 0 1	4 5 ± 0 3
Cyclic AMP change	8 1 ± 1 7***	1 4 ± 0 4 × **	1 0 ± 0 3**	1 5 ± 0 5*
Theophylline change	18 2 ± 3 8***	1 7 ± 0 6*	1 7 ± 0 4**	1 4 ± 0 4**
Cyclic AMP + Theophylline change	47 5 ± 5 7***	3 5 ± 0 6***	2 5 ± 0 7**	7 3 ± 0 6***
Significance of potentiation>	**	N S	N S	**

interest to study whether theophylline could reproduce any of the metabolic actions of noradrenaline or the other hormones tested In a concentration of 4.5×10^{-4} g/ml, theophylline significantly stimulated the oxygen consumption and the lactate, glycerol and FFA productions of brown adipose tissue (Table III) Theophylline failed however, to reduce the ATP, CrP and G 6-P contents of the tissue Sotalol diminished the effect of theophylline on the oxygen consumption and lactate production

Influence of cyclic AMP, dibutyryl cyclic AMP and cyclic AMP + theophylline on the metabolism of brown adipose tissue An important link in the chain of evidence which might explain the metabolic actions of noradrenaline as a consequence of increased cyclic AMP formation was to demonstrate that cyclic AMP itself could stimulate these metabolic processes We found that addition of cyclic AMP in a fairly high concentration (4.5×10^{-6} moles/ml), *i e* about 1000 times higher than the intracellular concentration moderately stimulated the oxygen consumption and the lactate, glycerol and FFA productions to about the same extent as noradrenaline in a concentration of 5×10^{-8} g/ml (Table III) Cyclic AMP failed however, to reduce the ATP concentration, which instead was significantly increased, neither did it influence the G 6 P content

It is recognized that cyclic AMP penetrates the cell membrane rather slowly and is rapidly hydrolyzed by phosphodiesterase (Robison *et al* 1965) As dibutyryl cyclic AMP is a more stable compound (Posternak *et al* 1962), we tested this substance in a 10 times lower concentration than cyclic AMP and found that it stimulated the oxygen consumption and FFA release to the same extent as cyclic AMP The calorigenic action of dibutyryl cyclic AMP was not influenced by sotalol but its FFA mobilizing effect was blocked (Table II) In high concentrations adrenergic β receptor blocking agents block the action of cyclic AMP on lipolysis (Aulich *et al* 1967)

If the rate of penetration of cyclic AMP was too slow for a sufficiently high intra cellular concentration to be built up it seemed possible that an inhibition of the enzymatic hydrolysis of cyclic AMP by theophylline would increase its effect We found that cyclic AMP and theophylline in combination gave a potentiated calorigenic action whereas their effects on the glycerol and lactate productions were addi

AMP + theophylline on oxygen consumption lactate and glycerol production and the ATP CrP
the sum of the separate effects of cyclic AMP and theophylline was less than their combined effect

Content of (μmoles g)

ATP	CrP	ADP	AMP
6	6	5	5
0 10±0 01	0 15±0 04	0 15±0 05	0 09±0 02
0 08±0 02*	0 07±0 03	0 00±0 02	0 04±0 02*
0 01±0 03	0 02±0 02	—0 06±0 03	—0 01±0 02
—0 10±0 02**	—0 13±0 02**	—0 04 0 03	0 00±0 02
**	**	N S	N S

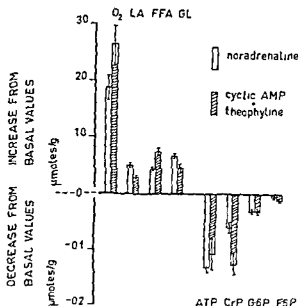


Fig 3 Comparison between the metabolic actions of noradrenaline (5×10^{6} g/ml) and cyclic AMP (4 5 µmoles/ml) + theophylline ($4\,5 \times 10^{4}$ g/ml) on the oxygen consumption (O_2), lactate production (LA), FFA release (FFA), glycerol production (GL), ATP content (ATP), CrP content (CrP), glucose 6 phosphate (G 6 P) and fructose 6 phosphate (F 6 P) Mean ± S E M of 6—8 tests

tive (Table III) In combination, cyclic AMP and theophylline also reduced the ATP, CrP and G-6-P contents of the tissue (Table III) The metabolic actions of cyclic AMP and theophylline were both qualitatively and quantitatively similar to the actions of noradrenaline in a concentration of 5×10^{6} g/ml (Fig 3)

There was a possibility that cyclic AMP after hydrolysis to 5'AMP, acted as a phosphate acceptor and thereby stimulated the oxygen consumption 5'AMP in the same concentration as cyclic AMP ($4\,5 \times 10^{6}$ moles/ml) had no significant effect on the oxygen consumption of brown adipose tissue but in a concentration about 6 times higher (3×10^{5} moles/ml) it stimulated the O_2 consumption to the same extent as cyclic AMP The differences in effects might be attributable to variation in the rate of penetration To study this possibility further we determined the effect of cyclic AMP on the 5'AMP content of the tissue and found a significant increase (Table III) As there was no change in the ADP content and an increase in the ATP content, it is very probable that cyclic AMP was the source of the 5'AMP In the presence of theophylline the rise of the 5'AMP after addition of cyclic AMP was blocked As theophylline at the same time potentiated the calorigenic action of cyclic AMP it is improbable that this effect was dependent on an increased 5'AMP formation from cyclic AMP

Influence of noradrenaline and cyclic AMP + theophylline on the phosphorylase activity of brown adipose tissue The classic action of cyclic AMP is to mediate the phosphorylase activating effect of the catecholamines (Sutherland *et al* 1968) It was therefore of interest to study how noradrenaline influenced the phosphorylase activity of brown adipose tissue and whether cyclic AMP could mimic its action Noradrenaline (5×10^{6} g/ml) increased both the total activity (in the presence of

0.001 M AMP) and the phosphorylase *a* activity (in the absence of AMP) When an attempt was made to reproduce these effects by adding cyclic AMP, it was found that cyclic AMP alone had a rather weak action In the presence of cyclic AMP+ theophylline and after 30—60 min of incubation there was however, a significant increase of both the phosphorylase *a* and the total activity

Discussion

The calorigenic and other metabolic actions produced by noradrenaline, 5 HT and ACTH in brown adipose tissue made this tissue a suitable object for comparing the similarities and dissimilarities between the effects of these hormones and cyclic AMP We found that

(1) Noradrenaline increased the cyclic AMP content of brown adipose tissue, thus satisfying the first of our criteria discussed in the introduction

(2) The metabolic actions of ACTH and 5 HT in brown adipose tissue were very like those of noradrenaline and our second question was at least partly fulfilled Butcher *et al* (1968) found that both hormones stimulated the cyclic AMP formation in white adipose tissue The metabolic actions of ACTH were almost identical with those of submaximal concentration of noradrenaline There was, however a difference between the actions of 5 HT and noradrenaline, 5 HT being the more potent calorigenic agent of the two, the reverse was found regarding the glycerol production The observation by Vaughan (1966) that 5 HT, like noradrenaline, stimulated the phosphorylase activity in white adipose tisue but had a very weak lipolytic action is of interest in this connection In our experiments noradrenaline and 5 HT were tested on tissue from different animals which may to some part explain the differences found

(3) Regarding our third question, sotalol almost completely blocked all metabolic actions of noradrenaline and 5 HT and partly those of ACTH We have found sotalol to inhibit the elevatory effect of adrenaline on the cyclic AMP formation in rat diaphragm Higher concentrations of sotalol were however needed to inhibit the metabolic actions of noradrenaline in brown adipose tissue Further studies are therefore indicated to ascertain if the inhibition was dependent on a specific adenyl cyclase blocking action of sotalol

(4) Theophylline in a concentration which inhibited the hydrolysis of cyclic AMP to 5'AMP produced most of noradrenaline's metabolic actions in brown adipose tissue An exeption was that theophylline failed to decrease the ATP and CrP contents of the tissue In combination with cyclic AMP however theophylline did produce these effects a finding which may indicate that theophylline alone raised the intracellular cyclic AMP concentration too little to induce this action.

(5) In Fig 3, qualitative and quantitative comparisons are made between the actions of noradrenaline and cyclic AMP+theophylline in the brown adipose tissue Qualitatively the resemblance is very close but quantitatively there are some dissimilarities The tests were performed on tissue from different animals, however,

which may explain the variation One possible explanation for the resemblance might be that the same receptors were stimulated by noradrenaline and cyclic AMP+ theophylline, or that the latter substances liberated bound noradrenaline from the tissue Brown adipose tissue is rich in noradrenaline (Wirsen 1964) As theophylline+ cyclic AMP still stimulated the metabolism after pretreatment of the animals with an adrenergic neuronic blocking agent (betanidine 10 mg/kg) this explanation is hardly likely

A weak point in these tests was that cyclic AMP had to be added in an extracellular concentration about 1000 times higher than was found intracellularly after noradrenaline treatment It is however well known that nucleotides and especially cyclic AMP penetrate the cell membrane slowly and are rapidly hydrolyzed by phosphodiesterase (Robison *et al* 1965)

An interesting question is the relationship between the different metabolic actions of noradrenaline and the mechanism of its calorigenic action in brown adipose tissue The reduction of the content of high energy phosphate compounds in the tissue was probably of importance

According to the classic concept, the rate of oxygen consumption in tissues is to a large part regulated by the availability of phosphate acceptors (ADP) and inorganic phosphate Our results may to some part be explained on this basis Noradrenaline 5 HT ACTH and cyclic AMP+theophylline all reduced the ATP content (probably thereby increasing the availability of ADP and P) and stimulated the oxygen consumption Cyclic AMP alone increased the 5'AMP content of the tissue and at the same time increased the ATP content of the tissue and at the same time increased the ATP content (Table III) and may by furnishing phosphate acceptors have increased the oxygen consumption Findings which at present cannot be explained on the basis of increased phosphate acceptors are that theophylline stimulated the oxygen consumption markedly without any effect on the ATP content (Table II) Instead it tended to decrease the ADP content of the tissue The ADP content in the tests with cyclic AMP+theophylline was significantly lower (−0 016±0 015 mole/g P<0 02) than in those tests with theophylline alone despite the fact that the increase of the oxygen consumption was potentiated by theophylline Another hypothesis which is in better agreement with these findings is that ATP in a high concentration had an inhibiting influence on some key reaction in the metabolic control This inhibiting influence was reduced either if the ATP concentration was decreased or in the presence of cyclic AMP The activity of phosphofructokinase seems at least partly to be regulated according to these principles (Mansour 1966) The possibility that the activity of other enzymes is regulated according to the same principles may be considered

It has been suggested that the calorigenic action of noradrenaline in brown adipose tissue is caused by an uncoupling effect of the oxidative phosphorylation by FFA released as a consequence of the lipolytic action of the hormone (Reed and Fain 1968) In the present study 5 HT had a stronger calorigenic action than noradrenaline, despite the fact that the lipolytic effect of 5 HT measured from the

glycerol production, was smaller than that of noradrenaline In further experiments we found that selective blockade of the lipolytic effect of noradrenaline by nicotinic acid did not inhibit its calorigenic effect in brown adipose tissue (Beviz *et al* 1968) or in the cold adapted rat *in vivo* (Jacobsson 1971) These observations do not support the assumption of a causal relationship between the lipolytic and calorigenic action of noradrenaline under the studied experimental conditions

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Blood Flow in the Calf Muscle of Man during Heavy Rhythmic Exercise

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Abstract

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ump' in the dependent legs despite the huge flow during the heavy rhythmic exercise This would result in a con iderable gain in local perfusion pressure and hence in maximal flow capacity

In a recent series of experiments Folkow, Gaskell and Waaler (1969 1970) analyzed in detail the effects of rhythmic exercise on arterial inflow of blood in a working muscle as well as the effects on local arterial and venous pressures They used the hind limb of the cat as a model and produced maximal exercise hyperemia by stimulating the sciatic nerve each second with trains of impulses lasting 200—300 msec a stimulation pattern that mimics natural running It was shown that there is virtually no inflow of blood during the contraction phase whereas each contraction of the muscle is accompanied by an intense spurt of venous outflow Between c tractions on the other hand there is very little venous outflow while arterial in is maximal

Further maximal blood flow capacities were compared in the exercising and ing leg muscles when the leg was placed either at heart level or some 35 cm below heart (leg down position) in order to determine the influence of incre vascular transmural pressure The free flow' in the post-exercise situation

largely the same in the two positions, indicating that the maximally dilated vessels were already fully stretched. Rhythmic exercise at heart level caused a moderately reduced maximal flow as a result of the inflow obstruction during about one third of the cycle. However, the same exercise in the "leg-down" position increased flow almost in direct proportion to the raised transmural pressure and maximal flow could now even be larger than that during the post exercise maximal hyperemia. Further, in this situation rhythmic exercise implied a marked lowering of mean venous pressure during the relaxation phase of the cycle, despite the huge inflow of blood into the veins, resulting in a corresponding gain in regional effective perfusion pressure. This gain in pressure head, and hence in flow, was ascribed to the pumping action of the muscles on the distended veins in association with the presence of competent venous valves.

The main intention of the present study was to investigate the effect of the leg position on blood flow in the calf muscles of man during heavy rhythmic exercise, since vascular transmural pressure is here markedly increased in the upright position. One would expect that the mechanism described by Folkow, Gaskell and Waaler may correspondingly increase the maximal flow capacity in dependent muscles of man during heavy rhythmic exercise. If so, this would markedly affect not only regional blood supply of the lower limbs in situations of maximal exertion but also cardiac output and general hemodynamics, since the major fraction of the muscle mass is placed below the heart level in exercising man.

Methods

The experiments were carried out on six healthy men, *178—190* cm tall. In a *first series* of experiments they performed heavy rhythmic exercise with their right calf muscles making plantar flexions against a strong spring resistance at a frequency of 1 per sec. The intensity of this work was such as to rapidly produce pain as a result of "relative ischemia" in the calf muscles, a situation where complete vascular relaxation in all probability had been attained. The subjects were first carefully instructed and trained to make fast brief contractions lasting approximately 0.3 sec and to fully relax their [illegible] the situation during e.g. running. They were plac[illegible]ing continued rhythmic exercise be shifted from [illegible]tion at a 60° angle with the horizontal plane. Continuous estimation of calf blood flow (see below) was made during this procedure.

In a *second series of experiments the subjects performed heavy exercise during* complete calf ischemia (cuff occlusion) until intense ischemic pain was experienced. Exercise was then stopped, the cuff pressure was suddenly released during complete muscular relaxation and calf blood flow was estimated during the period of maximal post exercise hyperemia. This type of experiment was first performed in the horizontal position, then twice in the "leg down" position and finally in the horizontal position again.

In a *third series* of experiments heavy rhythmic exercise was performed and immediately followed by a period of maximal post exercise hyperemia. Calf blood flow was continuously estimated. This type of experiment was first done in the horizontal and then in the "leg down" position.

Calf blood flow was estimated by following the wash out of ^{133}Xe locally injected into the calf muscles (*Lassen*, Lindbjerg and Munk 1964). Approximately 0.2 ml of a saline solution containing the radioactive isotope (0.5—1 mC/ml) was injected into the gastrocnemius muscle using a thin needle (outer diameter 0.4 mm). The [illegible]emission of ^{133}Xe was registered by a [illegible] relation [illegible] meter [illegible] 511)

Background was subtracted from the recorded wash-out curves and the counts per min were plotted *versus* time on semilogarithmic paper. These wash-out plottings were usually linear during the comparatively brief experimental series of the present study. Each of the 2, or 4, procedures in each experimental series lasted 2—4 min. From the half time value ($t_{1/2}$) of the uniexponential wash-out of radioactivity, the k value, depicting the rate of wash-out, and closely reflecting the rate of blood flow, can be calculated using the formula

$$k = \frac{\ln 2}{t_{1/2}}$$

Arterial blood pressure was recorded at heart level by the Korotkow method and the pulse rate was intermittently counted. Mean arterial blood pressure (MABP) at heart level was approximated, using the formula:

$$MABP = \text{diastolic pressure} + \frac{\text{pulse pressure}}{3}$$

The k value was divided by the mean arterial blood pressure and the changes in k/MABP, induced by the different experimental procedures, were compared. The change of k/MABP, was also expressed in per cent of control.

In the course of the present study it was frequently observed that the rate of wash-out

and second series of experiments) and then in combination (third series). Each experimental run in the three series was performed during wash out from one and the same tracer depot. Furthermore, in the second series of experiments the subject was titled first from supine to "leg down" position and then from a "leg down" to supine position, to cancel out the possible error inherent in estimating the blood flow from different sections of the wash out curve from a given tracer depot.

The arterial pressure at the calf level in the "leg-down" position was calculated by adding the vertical distance from the heart to the calf, expressed in mm Hg, to the mean arterial pressure measured at the heart level. Assuming that central venous pressure was close to zero, the same pressure addition could be made on the venous side to give the regional venous pressure in the "leg down" position during resting steady state. The average increase in trans mural pressure amounted to 65—70 mm Hg.

Results

In the *first series* of experiments heavy rhythmic exercise was continuously performed while the effect of the leg position on calf muscle blood flow was studied in terms of the changes in k/MABP value. Two series of recordings were performed in each subject. After a period of approximately 4 min of heavy rhythmic exercise in the supine position, the subject was tilted to a 'leg-down' position during continuous work and recording of the ^{133}Xe washout. Tilting increased k/MABP from a mean value of $(46 \pm 4.8) \times 10^{-4}$ (mean ± SEM, n = 12) in the supine position to $(74 \pm 8.9) \times 10^{-4}$ (n = 12) in the 'leg-down' position. This represents a mean increase of about 60 per cent (Fig. 1 left panel). In the supine position most subjects became tired and even experienced pain in the working muscle which was rapidly relieved by tilting to the "leg-down" position. At the same time the subjects found it much easier to perform heavy rhythmic exercise. Some reductions of heart frequency and systolic blood pressure were also observed with the shift of body position.

In the *second series* of experiments the ^{133}Xe wash out was studied in the phase of

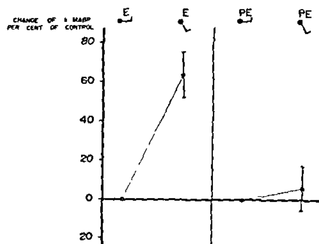


Fig 1 *Left panel* The effect of tilting the subject from supine to "leg down" position on calf muscle blood flow during maximal rhythmic exercise (E), expressed as per cent change of the k value of xenon wash out divided by mean arterial blood pressure (MABP) at heart level (k/MABP) Bars indicate ± S E M (n = 12)

Right panel The effect of the two positions supine and "leg down", on calf muscle blood flow during post exercise hyperemia (PE) expressed as per cent change of k/MABP The registration on each subject was first made in the supine position followed by two measurements in the leg down position and finally once again in the supine position Bars indicate ± S E M (n = 12)

maximal post exercise hyperemia in the two limb positions immediately following exhausting work during ischemia until pain developed In the supine position the average k/MABP value was $(55 \pm 4.0) \times 10^{-4}$ (n = 12) and in the leg down position it was $(58 \pm 8.0) \times 10^{-4}$ (n = 12) There is no statistically significant difference between these two values (see also Fig 1 right panel) This suggests that the maximally dilated resistance vessels were already fully stretched so that no further distension occurred in the leg down position

In the third series of experiments maximal blood flow was studied in the two positions not only during but also immediately after heavy rhythmic exercise First the ^{133}Xe wash-out was recorded in the supine position during the same heavy exercise as described above for approximately 3 min Then a cuff around the thigh was slowly inflated to about 200 mm Hg to produce complete ischemia in the calf which of course stopped the tracer wash out and the subject worked until ischemic pain was experienced Exercise was then stopped the cuff pressure was suddenly released and the ^{133}Xe wash out was recorded for 1 min during the intense postexercise hyperemia In the *supine* position the post exercise hyperemia was somewhat larger than the exercise hyperemia *where inflow was obstructed for about 0.3 sec each second* The average k/MABP values for exercise and postexercise hyperemia were $38 \pm 6.0 \times 10^{-4}$ (n = 10) and $(44 \pm 9.2 \times 10^{-4}$ (n = 10 respectively Rhythmic exercise was again resumed but now in the *leg down* position During this time the ^{133}Xe wash-out was recorded for 2—3 min and followed by a similar recording during the phase of postexercise hyperemia In the leg down position the wash-out was markedly enhanced during the period of heavy rhythmic exercise The mean k/MABP value during exercise was now $(57 \pm 9.9 \times 10^{-4}$ (n = 10 as compared with $(37 \pm 5.0) \times 10^{-4}$ (n = 10) in the subsequent phase of postexercise hyperemia The increase

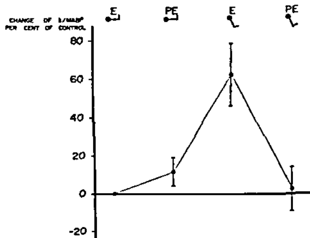


Fig 2 Changes in blood flow (k/MABP) during maximal exercise hyperemia (E) and post-exercise hyperemia (PE) in supine and "leg down" positions The alterations are expressed in per cent of the xenon wash-out registered in supine position during exercise The measurements were made on one and the same depot of ^{133}Xe figure Bars indicate ±S E M (= 10)

of wash-out during the period of exercise in the leg-down" position was, as calculated separately for each subject, approximately 60 per cent above the corresponding value in the supine position (Fig 2)

Discussion

It was demonstrated in the present study that calf muscle blood flow in man is markedly increased during standardized heavy rhythmic exercise when the subject is tilted from the supine to the "leg-down" position It can be argued that such an increase might be explained by a mere distension of the maximally relaxed resistance vessels in the dependent leg, resulting from the increased transmural pressure Even a slight increase of internal radius of the arterioles will profoundly increase flow as this is proportional to the fourth power of the radius It has, however, been shown that maximally relaxed resistance vessels are almost fully stretched at the normal pressure levels existing in the supine position Further increases of transmural pressure do not significantly increase the dimensions of the resistance vessels, at least not in cats This suggests that the "rigid jacket" of the vessels is already reached (Folkow and Lofving 1956) This conclusion was also supported by the present results on man since blood flow during maximal postexercise hyperemia is about the same in the supine and the 'leg-down positions On the other hand a shift from the supine to the 'leg-down" position during heavy rhythmic exercise increased the maximal blood flow capacity in the calf by about 60 per cent with an average increase of vascular transmural pressure in the calf of 65—70 mm Hg

The model experiments on cats by Folkow *et al* (1970), where local arterial and venous pressures were directly measured as well as phasic blood flow strongly suggest that this considerable gain in maxmial flow capacity during exercise in the "leg-down' position is, indeed, a result of a lowered mean venous pressure during the

relaxation phases, as caused by the action of the "muscle pump" This creates a corresponding gain in regional pressure head, since local arterial pressure remains increased as a result of the increased hydrostatic load As vascular distensibility is of little or no relevance at these pressure levels and as pressure and flow are linearly related according to Poiseuilles law, it follows that the effective pressure head for the vascular bed of the exercising calf must have increased in proportion to the flow, i e about 60 per cent In the present experiments on man the hydrostatic pressure was estimated to be increased some 65—70 mm Hg in the "leg-down" position on both the arterial and venous sides during resting steady state It follows that heavy rhythmic exercise can keep mean venous pressure markedly reduced during the relaxation period by the preceding action of the "muscle pump", as long as the venous valves are competent This occurs despite the huge inflow into the venous capacitance vessels during the relaxation phases The consequent gain in effective pressure head during the relaxation phase is so large (about 60 per cent) that the hydrostatic increase of venous pressure (65—70 mm Hg) must as a mean have been kept decreased by at least 80—90 per cent during the relaxation periods, as the result of the muscle pump and its emptying of the venous capacitance vessels during the contraction periods

The heavy rhythmic exercise in this study was performed in such a way as to mimic natural running Thus each contraction lasted 0 25—0 3 sec with a relaxation phase of 0 70—0 75 sec The results obtained may therefore help to explain the discrepancy between the maximal muscle blood flow (calf or forearm) measured in the supine position in humans (40—50 ml/min × 100 g tissue) and the roughly predicted maximal skeletal muscle blood flow of 20— 30 l/min delivered to 30—40 kg of muscle during heavy rhythmic exercise in well trained subjects The lower the position of the muscles in relation to the heart region the better the chances for a substantial gain in effective perfusion pressure and hence in maximal regional flow A gain of 35—45 per cent may be expected in the thighs and of 50—60 per cent in the calf regions in subjects of normal height

It is well known that patients with insufficient veins in the legs have reduced aerobic work capacity when tested e g on an ergometer bicycle (Arenander 1960) This has usually been explained as a result of an enhanced pooling of blood in the legs which reduces venous return to the heart and hence limits cardiac output However, the present results suggest that the reduced capacity for aerobic exercise may be mainly a consequence of a less efficient lowering of pressure in the varicose veins between contractions This correspondingly would reduce the gain in perfusion pressure and regional blood flow as compared with normal subjects Studies to test this hypothesis are in progress in this laboratory

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Acta physiol. scand. 1971. 81. 164—169

From the Renal Clinic, S t Eriks Sjukhus, the Department of Physiology, Karolinska Institutet and the Department of Clinical Chemistry, S t Eriks Sjukhus Stockholm Sweden

Electrolytes in Leg and Neck Muscles of Rats during Ontogeny

By

J Bergstrom, J Boethius and E Hultman

Received 22 June 1970

Abstract

Bergstrom, J, J Boethius and E Hultman *Electrolytes in leg and neck muscles of rats during ontogeny* Acta physiol scand 1971 *81* 164—169

The contents of sodium potassium magnesium chloride phosphorus and water were determined in the leg and neck muscle of 3 and 14 day old rats There were marked differences in electrolyte content between the two age groups and also between the leg and neck muscle of the 3-day old rat

The results indicate that there is a decrease in the amount of extracellular fluid during maturation The composition of the intracellular fluid also changes during development insofar as there is a marked decrease in its sodium content

The results indicated that the decrease in intracellular sodium roughly paralleled the rise in membrane potential It was therefore suggested that the developmental change in the intracellular sodium concentration was secondary to a decrease in the sodium permeability of the muscle cell membrane or to a change in the active transport of sodium across the membrane

The electrolyte composition of the muscle tissue changes during ontogeny (Dickerson and Widdowson 1960 Luff and Goldspink 1970) This change in muscle electrolyte is to a large extent secondary to a change in the relative proportions of the extra and intracellular fluids in the muscle tissue However also the intracellular ionic composition changes during ontogeny Thus Vernadakis and Woodbury (1964 showed that in the gastrocnemius muscle of newborn rats the intracellular sodium content is about twice as high as in 14-day old rats

In previous publications (Boethius 1969 1970 it was shown that in the rat there was an increase in gastrocnemius membrane potential at about the time when the above mentioned change in intracellular sodium content occurred in this muscle Within 5 days the membrane potential rose from the previous constant value of 60—70 mV up to the adult value of 80—90 mV When the potential development was studied in different muscles it was found that the potential rise occurred at different times in the different muscle groups In neck muscles the potential rise took place around birth, in the sartorius muscle it occurred between the 2nd and 7th day, and in the gastrocnemius muscle between the 5th and 10th day

In the rat gastrocnemius muscle the increase in membrane potential and the decrease in intracellular sodium thus occurs at about the same time This fact suggests that a developmental change in the muscle cell membrane may underlie both phenomena In order to obtain further evidence on this possibility the present study was performed on the electrolyte composition of the gastrocnemius and neck muscle of young rats

Methods

3 and 14 day old rats were anesthetized with ethyl ether Pieces of muscle tissue from neck and gastrocnemius muscle were rapidly removed Duplicate specimens from each muscle were immediately dissected free from visible fat and connective tissue and weighed on a Cahn electrobalance The specimens weighed about 5 to 15 mg each

Water and electrolytes were analyzed by previously described methods neutron activation analysis being used for determination of sodium potassium chloride and phosphorus (Bergstrom 1962) and atomic absorption flame photometry for (duplicate) potassium and magnesium determination (Beroniade Bergstrom and Hultman 1970) The extracellular and intracellular water content in the biopsy specimens were estimated according to Graham *et al* [illegible]

tent and a Donnan factor) Knowing the proportion of extracellular water and the total and extracellular concentrations of an electrolyte finally, the intracellular concentration of that electrolyte can be calculated On the basis of previous experiments (Boethius 1969 1970) the value chosen for the membrane potential of the gastrocnemius muscle of 3-day old rats was 65 mV In the gastrocnemius muscles of 14-day old rats and in the neck muscles from rats of both ages this chosen value was 85 mV Average values of plasma electrolyte determinations from rats in similar age groups (Vernadakis and Woodbury 1964) were used in the calculations

The procedures thus yielded four groups of values for each electrolyte In the case of the derived values (see below) these groups were subjected to a simple analysis of variance If the probability that the values were derived from the same population was less than 5 % the differences between values from muscles of the same age and kind were evaluated with t tests

Results

The muscle samples were taken from the gastrocnemius and neck muscles of 3- and 14 day old rats These age groups were selected since in the 3 day old rats the gastrocnemius membrane potential has not started to increase whereas the potential of the neck muscles has already reached adult values (Boethius 1970) In the 14 day old rat on the other hand both neck and gastrocnemius muscle have membrane potentials of adult magnitude

The results of the analysis can be seeen in Table I There is a general difference in electrolyte composition and water content between the muscles of the 3 and 14 day old rats There are also obvious differences between the gastrocnemius muscle and the neck muscle in the 3 day old rat

Marked developmental changes are seen in the total water content and the concentrations of the mainly extracellular sodium and chloride ions The water content and the contents of sodium and chloride are highest in the gastrocnemius muscle of the 3 day old rat The corresponding values of the neck muscle are lower than those of the gastrocnemius but higher than those of adult muscles At 14 days the

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In previous publications (Boethius 1969 1970) it was shown that in the rat there was an increase in gastrocnemius membrane potential at about the time when the above mentioned change in intracellular sodium content occurred in this muscle Within 5 days the membrane potential rose from the previous constant value of 60—70 mV up to the adult value of 80—90 mV When the potential development was studied in different muscles it was found that the potential rise occurred at different times in the different muscle groups In neck muscles the potential rise took place around birth, in the sartorius muscle it occurred between the 2nd and 7th day, and in the gastrocnemius muscle between the 5th and 10th day

14-day old rats							
Gastrocnemius muscle				Neck muscle			
n	x	SD	SE	n	x	SD	SE
6	6.12	4 04	1 65	6	20 90	21.38	8.73
6	433	8 32	3 40	6	420	11 94	4 88
6	13 45	1 46	0 60	6	13 03	1 67	0 68
6	17 11	1 97	0 60	6	16 44	2 19	0 89
6	46 50	2.06	0 85	6	45 40	1 01	0 41
3	10 01	0 85	0 49	3	9 31	0 56	0 33
3	39 97	0 85	0 49	3	37 13	1 01	0.58

day old rats In the gastrocnemius muscle of the 3-day old rat. however, there is significantly more intracellular water than in the other three muscles

The concentration of intracellular potassium is of about the same magnitude in all muscles On the other hand the concentration of intracellular sodium shows marked developmental changes Thus, the gastrocnemius muscle of the 3-day old rat has the highest intracellular sodium concentration (16 68 meq/l) In the neck muscle the *sodium concentration is lower (11 32 meq/l) and not statistically different from the* corresponding value at 14 days In the 14-day old rats the intracellular sodium content is roughly the same (7 61 and 7 20 meq/l) in the two muscles Similarly the content of intracellular sodium per dry weight of tissue is highest in the gastrocnemius muscle of the 3 day old rats (6 09 meq/100 g FFS) and appreciably lower (3 6 meq/100 g FFS) in the neck muscle On the 14th day the values have decreased further *and reached roughly adult levels (2 32 and 2 54 meq/100 g FFS) in both muscles*

In addition to dry weight and intracellular water, the amount of intracellular cations was also referred to the content of phosphorus (*cf* Bergstrom 1962) In the case of the mainly intracellular potassium the total muscle potassium was used to calculate the ratio When the intracellular electrolytes are referred to phosphorus one gets roughly the same developmental patterns as when they are referred to the

14-day old rats							
Gastrocnemius muscle				Neck muscle			
n	x	SD	SE	n	x	SD	SE
6	101	12.97	5 29	6	98	14 34	5.85
6	332	15 46	6 31	6	322	8.52	3 48
6	2 54	0 75	0 31	6	2 37	0 65	0 27
6	7 61	2 10	0.86	6	7 20	1 97	0 78
6	139 88	4 67	1 91	6	140 89	4.54	1 86
3	0 049	0 013	0 008	3	0 050	0 008	0 005
3	1 15	0 097	0 074	3	1.21	0 040	0 073

TABLE III Levels of significance

	G—G	N—N	G—N_3	G—N_{14}
H_2O_e	0 1 %	0 1 %	0 1 %	n s
H_2O_i	1 %	n s	0 1 %	10 %
Na_i	0 5 %	n s	0 5 %	n s
$[Na]_i$	0 5 %	n s	2 5 %	n s
$[K]_i$	n s	1 %	10 %	n s
Na_i/P	2 5 %	n s	10 %	n s
K/P	10 %	n s	n s	n s

P values for differences between the values of the two gastrocnemius muscles (G—G), the two neck muscles (N—N), the gastrocnemius and the neck muscle of 3 day old rats (G—N_3), the gastrocnemius and neck muscle of 14 day old rats (G—N_{14})

amount of intracellular water The potassium phosphorus ratio thus remains constant in all four muscle groups of the present investigation (1 19—1 22) Also the change in intracellular sodium is reflected in this ratio the values of the 3 day old rat being 0 136 in the gastrocnemius muscle and 0 083 in the neck muscle In the 14 day old rat the ratio has reached the comparatively adult value of about 0 050 in both muscles

As mentioned above, the level of significance for differences between the four muscles was first tested with a simple analysis of variance and if this difference was significant at a level of 5 % t tests were also performed for the differences between the two muscles of one kind and the two muscles of one age i e four t tests in total for each parameter The results of the t tests are shown in Table III

Discussion

Embryonic muscle tissue is characterized by the fact that its total water content is higher than that of the adult muscle and that the relative amount of sodium and chloride is larger than in adult muscle (Dickerson and Widdowson 1960) From the results of the present investigation it can be seen that the muscles are more immature in the 3 day than in the 14 day old rat Furthermore the gastrocnemius is of a more embryonic type than the neck muscle in the youngest rats At 14 days no such difference as to chemical maturity can be detected

The content of sodium potassium and chloride has previously been determined in the gastrocnemius muscle (Vernadakis and Woodbury 1964 Hazlewood and Nichols 1969) and the semimembranous muscle (Novikova 1964) of postnatal rats The gastrocnemius values of the present investigation correspond essentially to what was reported in the previous investigations In the case of magnesium the present investigation showed that the amount of this ion was about the same in the four muscles Similar results have been obtained in other developing mammals (Dickerson and Widdowson 1960)

The phosphorus content of the 3 day old gastrocnemius muscle is somewhat higher than the corresponding value of the 3-day old neck muscle and of the 14 day

muscles The high value of the 3-day old gastrocnemius muscle may be due to a low concentration of intracellular protein which makes up the bulk of the dry weight

Similarly to what is proposed for the phosphorus concentration the difference in the amount of intracellular water between the 3 day gastrocnemius on the one hand and the other muscles on the other may be attributed to a low concentration of protein in the immature gastrocnemius cells

The concentration of intracellular potassium remained relatively constant in the investigated muscles This is in agreement with the results of Vernadakis and Woodbury (1964) The amount of intracellular sodium showed a marked decrease from the chemically most immature muscle—the 3-day old gastrocnemius—to the values of the 14-day old rats The intracellular gastrocnemius values for 3 and 14 days agree with those described by Vernadakis and Woodbury (1964) On the other hand, Hazlewood and Nichols (1969) calculated the concentration of intracellular sodium to be about 140 meq/l at birth However, one reason for this high value seems to be that they assumed the membrane potential to be as low as 23 mV

The decrease in intracellular sodium concentration suggests developmental changes in the muscle cell membrane as to its relative sodium permeability and/or to its active sodium transport That such a developmental change occurs is also indicated by the change in membrane potential (Boëthius 1969 1970) which occurs around the period when the decrease in sodium concentration takes place

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From the Division of Physiological Chemistry, Chemical Centre, University of Lund, Sweden

Intracellular Distribution of Phospholipases in the Rat Pancreas

By

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Abstract

Arnesjo, B *Intracellular distribution of phospholipases in the rat pancreas* Acta physiol scand 1971 *81* 170—175

The intracellular distribution of free and potential phospholipases (EC 3 1 1 4) has been studied using isopycnic gradient centrifugation of rat pancreatic homogenates 30 % of the free phopsholipase A^2 activity was found in the zymogen granules while only 15 % of the total phospholipase A activity was present there Tryptic digestion increased the phospholipase A activity 20 fold—an increase only in phospholipase A^2 activity The zymogen granules accounted for 30 % of the prophospholipase and revealed a specific activity (activity per mg protein) 3 to 5 times higher than those of the other cell components The intracellular distribution of prophospholipase is similar to lipase

Recently we proposed that at least 25 % of the enzymic activities hydrolysing the fatty acid ester linkages of lecithin are localized in the zymogen granules obtained by density gradient centrifugation of pancreatic homogenates from previously fasted rats (Arnesjo and Filipek-Wender 1968) This activity, however, was too small to account for the high activity in the duodenal contents (Vogel and Zieve 1960 Gjone *et al* 1966) Shortly afterwards de Haas *et al* (1968) reported evidence for the presence of a precursor of phospholipase A in porcine pancreas Trypsin converted this prophospholipase to the active enzyme by splitting off a heptapeptide It was reported that there was virtually no increase in the phospholipase A activity after tryptic digestion of human and porcine pancreatic juice Later, however, we found a tenfold increase of the phospholipase A activity of rat pancreatic juice when trypsin was added in amounts exceeding the trypsin inhibitory capacity (Arnesjo *et al* 1967) The presence of prophospholipase in rat pancreatic juice was independently shown by Belleville and Clement (1968) for porcine and human pancreatic juice The tryptically released phospholipase A activities in porcine pancreas and rat and human pancreatic juice were shown to hydrolyse only the β fatty acid ester linkage of lecithin (De Haas *et al* 1968 Arnesjo *et al* 1967 Belleville and Clement 1968

The present investigation was undertaken in order to study the intracellular distribution of free and potential phospholipase A activities in the rat pancreas

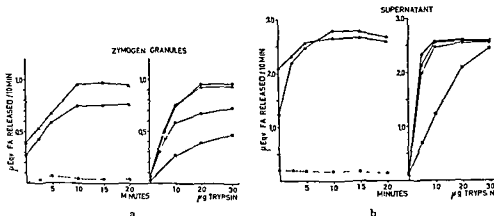
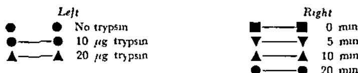


Fig 1 Influence of time and amount of added trypsin on the activation of prophospholipase in pancreatic homogenates Pancreatic homogenates (1 g tissue wet weight/5 ml 0 88 M sucrose) were centrifuged at $760 \times g_{av}$ for 10 min The resulting supernatant was then centrifuged at $4300 \times g_{av}$ for 20 min (Siekewitz and Palade 1958) Similar aliquots from the re-



Material and Methods

Male Sprague Dawley rats (AB Anticimex, Stockholm) were fasted overnight and then sacrificed by cervical dislocation Pancreatic homogenates (1 g tissue wet weight per 5 ml 0 88 M sucrose) were prepared and fractionated by density gradient centrifugation as previously described (Arnesjö and Filipek Wender 1968, Arnesjo and Grubb 1969) Lipase and phospholipase activities were assayed as previously described (Arnesjo and Filipek-Wender 1968) In the assay of free phospholipase activities an incubation period of two hours was used but this was shortened to 10 min when testing fractions activated by tryptic digestion Purified

... 15 ml chloro-
... ctivity of
... trometer

...) Fig 1 shows that this procedure gives maximal activation of prophospholipase present in rat pancreatic homogenates or subfractions Control experiments showed that an essentially linear relationship existed between the amount of tissues and the released phospholipase A activity within a range of 0—2 5 mg pancreatic tissue wet weight

Protein was measured according to Lowry *et al* (1951) as modified by Eggstein and Kreutz (1955) and phospholipid phosphorus by the method of Chen *et al* (1954)

TABLE I Percent distribution and recovery of phospholipase and lipase activities and of protein after equilibrium density centrifugation of rat pancreatic homogenates Homogenates [illegible]

Measured	Applied	Recovered	Recovery %	% of applied activity recovered at densities		
				1 240—1 201	1 201—1 175	<1 175
Phospholipase						
μeq FA released/ 2 hrs	4 03	—	—	15 7	1 8	82 5 (calc)
μeq β FA released/2 hrs	2 93	2 44	83 4	31 3	2 4	49 7
μeq FA released/ 10 min after tryptic digestion	9 49	8 64	87 3	29 5	6 5	50 44
Lipase						
μeq FA released/ 2 min	290 3	241 3	83 3	30 4	12 5	43 6
Protein mg	14 30	12 11	84 7	11 8	7 0	65 9

Results

As it is possible that several enzymic activities residing in fresh pancreatic homogenates are responsible for the release of fatty acids from micellar lecithin the total release and the release of the labelled fatty acid from ^{3}H β acyl lecithin were determined simultaneously As shown in Table I these two activities show different distribution after fractionation of pancreatic homogenates by isopycnic density gradient centrifugation The β fatty acid ester splitting activity was distributed as 30 and 50 % between the zymogen and membrane cell sap fractions respectively The total fatty acid splitting activity however, displayed only a small peak of activity in the zymogen granule fractions constituting 15 % of the total phospholipase A activity As previously reported (Arnesjo and Filipek-Wender 1968) the membrane-cell sap fractions were shown to contain a large amount of fatty acid, resulting in high blank values Therefore only experimental data concerning the zymogen granule and mitochondrial fractions are presented By comparison with the specific activity of the ^{3}H β fatty acid of the lecithin substrate (as determined by the method of Hanahan (1954) using *Crotalus adamanteus* venom) the specific activity of the fatty acids released by the zymogen granule fractions indicates that around 70 % of the activity responsible for the hydrolysis of micellar lecithin at pH 9 is of the phospholipase A^2 type Assuming the recoveries of phospholipase A activity as measured by the total release of fatty acids to be the same as measured by release of the ^{3}H β fatty acid from the labelled lecithin only 50 % of the activity present in the membrane cell sap fractions was calculated to be due to a phospholipase A^2 activity Only small quantities (max 5 % of ^{3}H β labelled lysolecithin were formed during the en

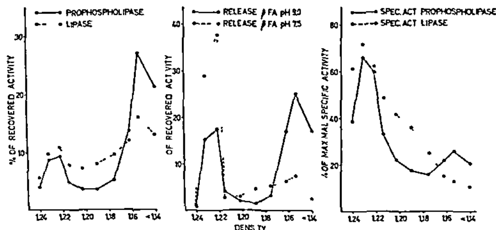


Fig 2 Distribution of total free and potential phospholipase A activities and of specific activities (activity/mg protein) for trypsinactivable phospholipase A after density gradient centrifugation of rat pancreatic homogenates The data represent the means of at least 2 experiments and are for reference compared with the same distribution of lipase

zymic reactions Therefore the predominant activity splitting the α fatty acid ester linkage of lecithin is of the lysophosphatidase type However, a splitting of the α fatty acid ester linkage prior to the β fatty acid cannot be excluded In the mitochondrial fractions no peak of either enzymic activity was observed

When the subcellular pancreatic fractions were incubated with micellar ^{3}H β acyl lecithin at pH 7 5 only small activities were found in the membrane fractions The zymogen granule fractions however, exhibited almost as high activities as those found with incubation at pH 9 (Fig 2) Virtually no activities could be demonstrated at pH 6

After tryptic digestion pancreatic phospholipase A activity was increased approximately 20 fold This increase was highest in the membrane cell sap fractions (Fig 1 and Table I) The trypsin activable phospholipase A activity was distributed among the subcellular components of the rat pancreas according to Fig 2 which figure also shows the distribution of lipase By comparison with lipase the prophospholipase is distributed more towards the membrane cell sap fractions but the percentages in the zymogen granule fractions were similar Pancreatic homogenates previously digested with trypsin released fatty acids from ^{3}H β acyl lecithin with almost as high a specific activity as the β fatty acid of the lecithin substrate

Discussion

The results reported here provide evidence for the existence of at least two phospholipase A activities in fresh pancreatic homogenates from previously fasted rats These activities hydrolyzing the ges of lecithin respectively,

TABLE I Percent distribution and recovery of phospholipase and lipase activities and of protein after equilibrium density centrifugation of rat pancreatic homogenates Homogenates [illegible]

Measured	Applied	Recovered	Recovery %	% of applied activity recovered at densities 1 240—1 201	1 201—1 175	<1 175
Phospholipase						
μeq FA released/ 2 hrs	4 03	—	—	15 7	1 8	82 5 (calc)
μeq β FA released/2 hrs	2 93	2 44	83 4	31 3	2 4	49 7
μeq FA released/ 10 min after tryptic digestion	9 49	8 64	87 3	29 5	6 5	50 44
Lipase						
μeq FA released/ 2 min	290 3	241 3	83 3	30 4	12 5	43 6
Protein mg	14 30	12 11	84 7	11 8	7 0	65 9

Results

As it is possible that several enzymic activities residing in fresh pancreatic homogenates are responsible for the release of fatty acids from micellar lecithin the total release and the release of the labelled fatty acid from ^{3}H-β acyl lecithin were determined simultaneously As shown in Table I these two activities show different distribution after fractionation of pancreatic homogenates by isopycnic density gradient centrifugation *The β-fatty acid ester splitting activity was distributed* as 30 and 50 % between the zymogen and membrane cell sap *fractions, respectively* The total fatty acid splitting activity however, displayed only a small peak of activity in the zymogen granule fractions, constituting 15 % of the total phospholipase A activity As previously reported (Arnesjo and Filipek-Wender 1968) the membrane-cell sap fractions were shown to contain a large amount of fatty acid, resulting in high blank values Therefore only experimental data concerning the zymogen granule and mitochondrial fractions are presented By comparison with the specific activity of the ^{3}H-β-fatty acid of the lecithin substrate (as determined by the method of Hanahan (1954) using *Crotalus adamanteus* venom) the specific activity of the fatty acids released by the zymogen granule fractions indicates that around 70 % of the activity responsible for the hydrolysis of micellar lecithin at pH 9 is of the phospholipase A^2 type Assuming the recoveries of phospholipase A activity as measured by *the total release of fatty acids* to be the same as measured by release of the ^{3}H-β-fatty acid from the labelled lecithin, only 50 % of the activity present in the membrane-cell sap fractions was calculated to be due to a phospholipase A^2 activity Only small quantities (max 5 % of ^{3}H β-labelled lysolecithin were formed during the en-

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Reactivity to Noradrenaline of Aortic Strips and Portal Veins from Spontaneously Hypertensive and Normotensive Rats

By

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Abstract

HALLBÄCK, M, Y LUNDGREN and L WEISS *Reactivity to noradrenaline of aortic strips and portal veins from spontaneously hypertensive and normotensive rats* Acta physiol scand 1971 *81* 176—181

To investigate whether vascular smooth muscle of spontaneously hypertensive rats, SHR, (Okamoto 1963) displays any hypersensitivity to noradrenaline (NA) as compared to that of normotensive control rats, NCR, helical aortic strips, alternatively portal vein segments, from a SHR and a NCR were mounted in the same organ bath, to which graded NA doses were added In [illegible] ty to NA of the vascular effector cells proper but may rather, as suggested by Folkow *et al* (1970) be due to an increased wall/lumen ratio of the SHR resistance vessels

At present three main mechanisms which alone, or in combination, may produce the increased flow resistance in essential hypertension are discussed First, there may be an enhanced vasoexcitatory influence (neurogenic, bloodborne or local) to which the arterioles respond in a normal way Second hypersensitivity of the vascular smooth muscles may cause an increased resistance response to normal vasoexcitatory influences Third some type of structural vascular change may be involved of such a nature that an increased resistance is established even at a normal level of smooth muscle activity

Folkow (1956) Folkow *et al* (1958) Conway (1963) and Sivertsson and Olander (1968) have shown that the regional flow resistance in forearm and hand of patients with essential hypertension is raised even at maximal dilatation It has also been demonstrated that the NA dose-response curve in the hypertensive subjects is steeper while the NA threshold is largely the same as in the normotensive controls (see also Sivertsson 1970) These findings in man with well-established essential hypertension suggest the dominant influence of a change according to the third alternative, mentioned above

Recent studies of the entire systemic vascular bed of spontaneously hypertensive rats, SHR, (Okamoto 1963) indicate that also in these animals flow resistance is significantly increased even at complete relaxation of the vascular smooth muscles as compared to normotensive control rats NCR (Folkow *et al* 1970 a) Further when NA was infused to the perfused hindquarters, the dose response curve of SHR was significantly steeper and the maximal pressor response was increased by some 40 %, while the 'threshold' sensitivity to NA (and other vasoconstrictor agents) was about the same as in NCR (Folkow *et al* 1970 b)

Thus, also these findings—obtained in what seems to be the best 'animal model so far of essential hypertension in man—suggest the presence of a structurally determined increase in wall/lumen ratio of the resistance vessels, presumably involving particularly the media, as also the maximal contractile strength was markedly enhanced These structural changes of the resistance vessels in SHR were, in fact, so pronounced that they may alone largely account for the raised flow resistance and blood pressure in the resting steady state

In vitro preparations of perfused mesenteric arteries from the same type of hypertensive rats were recently studied by Haeusler and Haefely (1970) and compared to similar preparations from normotensive control rats They interpreted their results as indicating that these larger arteries of the SHR displayed hypersensitivity to NA, but they also noted that the SHR arteries exhibited an increased maximal pressor response compared with the NCR ones However, the influence of a possible change of vascular wall/lumen ratio on dose response curves for blood vessels was apparently not considered in detail in this study

In order to investigate, by another type of approach whether the vascular smooth muscles proper of SHR really display any true hypersensitivity to NA an *in vitro* study was performed both on aortic strips and on sections of the portal veins from SHR and NCR By recording the responses of strips of vessels instead of the flow resistance changes in intact vessels where possible differences in the wall/lumen ratio will also effect the results (see *e g* Folkow 1956) the actual contractile properties of the vascular smooth muscles can be more directly measured For technical reasons aortic smooth muscle was used although such a preparation like that of the mesenteric arteries does not necessarily mirror the characteristics of the smooth muscle in the true resistance vessels In order to study the noradrenaline effects on a vessel which exhibits similar properties as the small precapillary resistance vessels in terms of spontaneous rhythmic activity and myogenic propagation the smooth muscle of the rat portal vein was studied as well

Method

The descending thoracic aorta and the portal vein from 10 SHR and 10 matched NCR were used in the present study *Each pair of animals were of similar age (7—9 months) size and sex* Mean blood pressure of anesthetized NCR and SHR of this age was 123 ± 4 mm Hg and 174 ± 9 mm Hg respectively

The animals were killed by a blow on the neck the abdomen was opened and the central part (1 cm long) of the portal vein was cautiously prepared free and put into oxygenated Krebs solution The thorax was opened and the lower part of the thoracic aorta was taken out and

transferred to a dissection bowl for cutting helical strips The sectioning of the aorta was carried out with an ordinary pair of dissection scissors and precaution was taken to keep the size of the strips from the two animals as similar as possible

After the preparation the aortic strips from SHR and NCR were mounted in the same muscle bath and the portal veins from the two animals in another bath both containing a Krebs solution (composition in mmoles/l NaCl 122 KCl 4 73 $NaHCO_3$ 15 48 KH_2PO_4 1 19 $MgCl_2$ 1 19 $CaCl_2$ 2 49, glucose 11 5 and $CaNa_2$-versenate 0 026) Throughout the experiment the solution was continuously bubbled with a gas mixture of 4 % carbon dioxide in oxygen and the temperature was kept constant at 38° C The arrangement to mount the two preparations to be compared in the same muscle bath secured identical environments and NA dosages One end of each vascular preparation was fixed to the bottom of the muscle bath while the [illegible]

[illegible] Mallov 1959 and Felper 1969) The portal veins were stretched to their approximate length in situ giving a passive tension of about 400 mg

[illegible] r preparations were allowed to recover [illegible] β adrenergic receptors which possibly [illegible] led to the bath to give a concentration [illegible] ashed out When the aortic strips were completely relaxed and the portal [illegible]

[illegible] ms for the highest doses given [illegible] was rinsed several times with fresh Krebs [illegible] ir resting tension for a period of 30 min [illegible] lose response curves were expressed as per cent of maximal tension which largely abolishes irregularities dependent of technical incidents and also the influence of differences in tension as expressed in mg

Results

The mean noradrenaline dose response curves from 10 successful experiments on preparations from SHR and NCR are illustrated in Fig 1 As is seen from Fig 1 practically identical dose response curves were obtained for both the aortic strips and the portal veins from SHR and NCR The ED_{50} value for the aortas i e the NA dose producing 50 per cent of the maximal tension was $2.8 \times 10^{-10} \pm 0.6 \times 10^{-10}$ g NA/ml for SHR and $2.5 \times 10^{-10} \pm 0.6 \times 10^{-10}$ g NA/ml for NCR The portal veins of the SHR showed an ED_{50} of $6.9 \times 10^{-8} \pm 0.8 \times 10^{-8}$ g NA/ml while it was $7.0 \times 10^{-8} \pm 1.2 \times 10^{-8}$ g NA/ml for the NCR Thus no difference in sensitivity between the SHR and NCR strips was noticed Even if the true threshold of the portal veins is difficult to determine exactly because of their spontaneous activity no difference whatsoever was noticed between the SHR and the NCR as far as their dose response curves are concerned

The SHR portal veins developed a maximal active tension of 510 ± 47 mg and the NCR ones 531 ± 29 mg ($n = 10$) the corresponding values for the aortic strips being 646 ± 63 mg and 637 ± 13 mg ($n = 10$) respectively

Discussion

Many investigators have observed enhanced pressor or resistance responses to intravenous or intraarterial infusions of vasoexcitatory agents in hypertensive subjects or animals as compared to normotensive controls In most cases however such observa

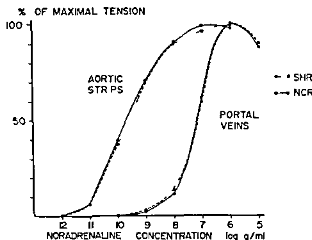


Fig 1 Noradrenaline dose response curves for aortic strips and sections of portal veins from SHR and NCR The responses are expressed as per cent of maximal tension

tions do not allow any differentiation between the extent of *smooth muscle* shortening to given vasoexcitatory stimuli *e g* a true hypersensitivity and such vascular hyperresponsiveness in terms of the *resistance* increases that may be due merely to a structural increase of wall/lumen ratio

To explain the often observed vascular hyperresponsiveness in hypertension the involvement of different combinations of bloodborne and neurogenic excitatory influences have usually been considered *i e* it is often taken more or less for granted that the response of the contractile elements proper should be changed and this may no doubt often be the case at least intermittently and/or in acute phases of the disease If however an adaptive increase of the wall/lumen ratio has occurred in the resistance vessels perhaps as a response to some more or less intermittent functional trigger load such as an increased blood pressure the equilibrium level and the range of constriction/vasodilatation of the resistance vessels will become reset to a higher level thus resulting in a type of vascular hyperresponsiveness where the smooth muscles in terms of sensitivity and extent of shortening may still respond in a perfectly normal way In fact some studies of regional vascular beds in man (hand and forearm) mentioned in the introduction suggest that this may be so during resting equilibrium in well-established essential hypertension

Studies along similar lines were recently started on spontaneously hypertensive rats SHR (Okamoto 1963) which seem to provide the best animal model so far for essential hypertension in man In such animals more extensive studies involving the entire systemic circulation or individual vascular circuits can be performed Thus it has been shown that the isolated hindquarters of SHR besides exhibiting an increased flow resistance even at maximal dilatation display a steeper dose response curve to NA an increased maximal pressor response while the threshold to NA is unchanged as compared to NCR (Folkow *et al* 1970 b) A comparison with two mathematically deduced resistance curves for two hypothetical resistance vessels identical in all

respects except for a 30 per cent increase of the media thickness encroaching upon the lumen in the 'hypertensive' vessel model, revealed a remarkable similarity between the experimental and the hypothetical "resistance curves"

Haefely and Haeusler (1970), studying the perfused mesenteric arteries of the same type of animals, arrived at the conclusion that the SHR arteries were supersensitive to NA However, they apparently did not consider in detail how dose response curves for vessels will be affected if *e g* the wall/lumen ratio in the SHR vessels were increased The use of *intact* vessels and their luminal responses for studies of vascular smooth muscle sensitivity to pressor agents involves some hazards whenever they differ in terms of wall/lumen ratio The reason is that an increased wall/lumen ratio will *per se* exaggerate the responses to vasoactive substances giving a steeper curve, a displacement of 'M_{50}' (50 % of the maximal response which in intact vessels is *not* identical with ED_{50} if the wall/lumen ratio is altered) to the left and also an increased maximal response This latter change was seen in the experiments of *Haefely and Haeusler and may indicate that the wall/lumen ratio of the* mesenteric artery preparation studied did differ (compare Folkow *et al* 1970 a, b) To exclude such influences the present study was performed on *strips* of smooth muscle and the responses were moreover, presented in per cent of maximal response

In vitro studies have been performed concerning the influence of vasoactive drugs on aortic strips from normotensive rats and rats with renal hypertension or hyper*tension due to desoxycorticosteron acetat (DCA) treatment (e g Redleaf and Tobian* 1958 Malloy 1959) In these studies the strips were stretched to a standardized passive tension level and graded NA doses were given to produce a dose response curve If anything the hypertensive vessels displayed a decreased sensitivity to NA Gordon and Nogueira (1962) on the other hand observed *increased* responses to equal amounts of NA when studying similar preparations However they used a technique *where a standardized NA dose was given to strips exposed to different levels of passive* sive tension level and graded NA doses were given to produce a dose response curve In a quite recent study of aortic strips from SHR Spector *et al* (1969) found that these strips were less responsive to NA and other vasoactive agents than those from NCR

In the present study no significant difference was observed in the NA sensitivity either for the aortic strips or *for the portal veins from SHR and NCR as judged by e g* ED_{50}

The fact that the aortic strips of both SHR and NCR proved to be more sensitive to NA than the portal veins from the same animals may be due to the fact that the rat portal vein appears to have a more extensive nervous supply and perhaps more "close" neuro-effector junctions than the aorta (Johansson *et al* 1970) Therefore the higher threshold *of the portal veins might mainly reflect a more efficient up*take mechanism for exogenous NA into the adrenergic nerve endings in these vessels

The present results on isolated strips of both multi unit and single unit (with myogenic activity) vascular smooth muscle from SHR and NCR are thus in agreement with the view that the characteristic differences between these animals as to

the 'resistance curves' of their perfused hindquarters (Folkow *et al* 1970 b) is not a matter of increased smooth muscle sensitivity or 'reactivity', but can be explained by an increased wall/lumen ratio in the SHR resistance vessels

We are most indebted to the Genetics Unit, National Institutes of Health Bethesda, USA for

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The Time Course of the Active State in Relation to Sarcomere Length and Movement Studied in Single Skeletal Muscle Fibres of the Frog

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Abstract

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[illegible] ing to a decrease in duration of the activity at the shorter length. The duration of [illegible] active state at 1.9 μ sarcomere spacing was 69 (range 51–80) per cent of the duration existing at 2.6 μ sarcomere length as determined in 7 different fibres. Movement (active shortening) *per se*, apart from the change in sarcomere length, affected the kinetics of the active state. An interval of free shortening interposed during an isometric contraction reduced the fibre's capacity to produce tension for the rest of the contraction period without changing the total duration of the mechanical activity substantially. The movement produced a relatively small effect as long as the active state was maximum; the depressant effect became greater and greater, however, the later the movement occurred during the decay phase of the active state. The nature of the length and movement dependence of the active state is discussed in the light of the calcium activator mechanism of the excitation-contraction coupling.

According to the analytical model advanced by A. V. Hill (1938) the active contractile unit of muscle operates in series with a passive elastic element. When the muscle is stimulated, the contractile unit is brought into an "active state" which is manifested externally as an overall shortening of the muscle or, if the movement is restrained, as a production of tension. Using Hill's analytical approach the actual capacity of the contractile unit to produce tension and motion (the intensity of the active state) can be experimentally defined at different times during the contraction.

Evidence obtained in previous studies on amphibian skeletal muscle suggests that the time course of the active state is dependent on muscle length. Thus, as demonstrated in the frog's sartorius muscle (Ritchie 1954, Ritchie and Wilkie 1958) an in

crease in resting length of the muscle is associated with a prolongation of the active state The more intimate relationship between the kinetics of the active state and muscle length is still unclear, however There is no clue from the previous work as to whether or not the observed changes in duration of activity involves any alteration of the rising phase of the active state Nor has it been possible to evaluate the active state curve in relation to the actual sarcomere length of the muscle

The present experiments have been aimed at elucidating the length dependence of the active state in more detail The analysis has been carried out on single skeletal muscle fibres of the frog and the approach used has made it possible to examine both the rising and falling phases of the active state over a wide range of sarcomere lengths The results have shown that neither the speed of development of the active state nor the rate, by which it decays, are markedly affected by changes in fibre length However, a decrease in sarcomere length is associated with a shift of the decay phase to an earlier time after the stimulus leading to an abbreviation of the active state at the shorter length It is pertinent that the duration of the active state is steadily decreased by reducing the sarcomere length irrespective of how the maximal tension output is affected by the length change

Certain observations in previous studies have indicated that the time course of the active state might also depend on the amount of shortening that occurs during the actual contraction period As has been demonstrated in both skeletal muscle (Jewell and Wilkie 1960) and myocardium (Brady 1966) the ability of the muscle to carry a load during a twitch is lost at an earlier time after the stimulus in the case that some shortening has occurred during the contraction Experiments were undertaken to further analyze this phenomenon The results have shown that the shortening process *per se*, quite apart from the change in sarcomere length affects the kinetics of the active state during contraction of the fibre Evidence will be presented demonstrating that movement of the fibre reduces the intensity of the active state but does not cause any appreciable change in the total duration of the mechanical activity Brief accounts of the results described in this paper have been presented earlier (Edman and Kiessling 1966 Edman 1968)

Methods

Preparation and mounting Single fibres were dissected from the ventral or dorsal heads of the semitendinosus muscle of R temporaria The dissection was usually carried out in the morning on the day of the experiment in a few cases the fibre was prepared the evening before and kept overnight in a cold room (2—4° C) No difference in results between fibres dissected on the same day or the evening before were observed The fibres were carefully cleaned from debris and only a small portion of the original tendons approximately 2 mm long and 0 5 mm wide was left at each end of the fibre Loops of polished stainless steel wire (diameter 100 μ)
[illegible] mately 1 mm
and 1 mm
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n The fibre
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transducer

Recording arrangement A schematic drawing of the experimental set up is given in Fig 1 The recording trough (A) was fitted on top of a lucite box The volume of the trough was 9

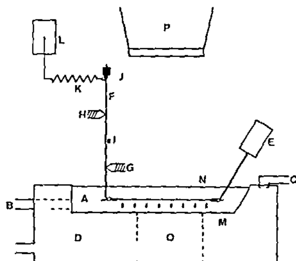


Fig 1 Schematic illustration of arrangement used in quick release experiments A Muscle fibre chamber B inlet for bath solution C suction drain D jacket for circulation of thermostatically controlled water-glycol mixture E tension transducer (RCA 5734) F isotonic lever G and H micrometer screws I releasable stop J fulcrum of isotonic lever K coil-spring for loading of lever L strain-gauge tension transducer M multielectrode assembly in muscle chamber N muscle fibre O light path P Zeiss Stereo II microscope fitted with cine camera

ml Ringer s solution was added through an inlet (B) at the end of the trough, a constant fluid level being achieved by suction drain (C) at the opposite end of the bath The bathing fluid

experiment

Tension was recorded by means of an RCA 5734 mechano electric transducer (E) fitted with a glass tube (length 25 mm outer diameter 2 mm) which was attached to the anode pin with Epoxy resin The end of the glass tube was bent into a fine hook The valve was mounted at an angle of 45° relative to the longitudinal axis of the trough and was clamped in a brass block fitted on an adjustable bracket An isotonic lever (F) was mounted at the other end of the trough The lucite block to which the lever was mounted could be moved along a slide by means of a screw The angular movements of the lever was controlled by micrometer screws (G H) in front of and behind the lever The back screw acted directly upon the lever the front screw controlled the position of an arm which extended at right angle from a vernier scale placed parallel to the trough With this arrangement there was enough space for a microscope tube fitted with a water-immersion objective to be inserted above the fibre (see below) A third stop (I) was used for quick release of the lever during active state recordings The stop consisted of an arm of solid brass mounted on the main switch of an electric relay Only the tip of the arm made contact with the isotonic lever Closing the relay produced an instantaneous release of the isotonic lever The relay was synchronized with the stimulation signal and could be operated at any pre set time during contraction by means of an electronic delay circuit

Isotonic lever The lever (length 45 mm) was mounted on a horizontal axis (J) which was fitted in steel bearings The lever and axis were both made of steel tubing (outer diameter 0 9 mm) except for the distal end of the lever, which was provided with a hook of steel wire diameter 0 5 mm) and the ends of the axis which had conical tips of polished hardened steel The lever was very carefully balanced by means of a counter weight to get the centre of gravity of the moving system close to the fulcrum The static friction of the lever was less than 1 2 ... the fibre was 44 mg Loading of ... (not illustrated) extending from ... lever was loaded by means of a ... ducer (L) and a point close to the fulcrum of the lever The distance from the fulcrum to the points of attachment of the ... carrier ampli ... electric trans ... cording trough

has been described above The signal from the transducer was displayed to one of the channels of a Tektronix 502 A oscilloscope A linear response was obtained for forces up to at least 400 dynes *i e* for the range covered in the present experiments The frequency response of the transducer with the glass tube attached to the anode pin was approximately 500 c/s

In some experiments the first derivative of the tension output was recorded by feeding the signal from the transducer into an RC circuit (time constant 0 5 msec) In such cases the original signal and its first derivative were displayed simultaneously on the two beams of the oscilloscope

The oscilloscope traces were recorded photographically on 35 mm film (Cientia 135) using a Cossor oscilloscope camera

Stimulation The fibre was stimulated by passing current through an assembly of platinum wire electrodes (M) spaced at 2 mm intervals along the length of the fibre The distance between the wires and the fibre was 1 5 mm The wires (0 125 mm diameter) were coated with a thin layer of Epoxy cement except for a 3 mm long portion facing the fibre The electrodes were arranged as alternate anodes and cathodes and care was taken to ensure that each pair of electrodes produced a supramaximal stimulus Square pulses of 1 msec duration were used A series of 4 pulses of appropriate frequency was given at 2 min intervals to produce an incompletely fused tetanus (for further information, see section A Results) After 10—15 such contractions a 1 sec train of pulses of a frequency of 40/sec was given to produce a completely fused tetanus The fibres were usually mounted 4 hrs before the experiment and were tetanized occasionally during that time Only fibres that survived many hours of continuous experimentation were used

Determination of sarcomere length The sarcomere spacing at rest was measured by direct microscopy at 800 times magnification using a water immersion objective (Zeiss 40 X N A 0 75 focal length 4 6 mm working distance 1 6 mm) and an ocular micrometer Sequences of about 20 sarcomeres were measured at different locations in the middle segment of the fibre (about 90 per cent of the fibre length), in which the sarcomere pattern is very nearly uniform (Edman 1966) A mean value of the sarcomere length was obtained from measurements of 5 to 6 different sequences The same procedure was repeated at four to five different settings of the isotonic lever It was checked that the same linear relationships between sarcomere spacing and setting of lever existed before and after the experiment

The film speed used was 64 f p s and the exposure time was 2 5 msec Changes in distance between the markers could be read after enlargement of the film records to an accuracy of 0 5 per cent The rationale of calculating the sarcomere length from overall movements of the fibre is given by the fact that the sarcomeres in the middle segment of the fibre exhibit a very uniform behaviour during activity under the experimental conditions used (Edman 1966 Cleworth and Edman 1969)

Measurements of the action potential Glass capillary electrodes filled with 3 M KCl were used for intracellular recording of the action potential Electrodes of about 20 Mohm resistance and tip potential of <5 mV were selected The electronic arrangement was similar to that used previously (Edman Grieve and Nilsson 1966) With a microelectrode of 20 Mohm the input time constant was about 150 μsec

Solutions A Ringer s solution of the following composition was used (mM) NaCl 115 5 KCl 2 0, $CaCl_2$ 1 8 NaH_2PO_4 Na_2HPO_4 2 0 pH 7 0 Glass distilled water was used for washing of the glass ware and for preparation of solutions The chemicals used were of analytical grade

Results

For the following study of the active state the conceptual model originally advanced by A V Hill for whole muscle has been used According to this model the muscle fibre is considered to act as a contractile unit in series with a passive elastic element The presence of parallel elastic components has been ignored in view of the fact that there is virtually no resting tension in the single fibre preparation at sarcomere lengths below 2 8 μ *i e* at the lengths studied in the present experiments The term

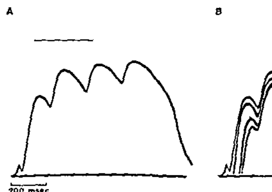
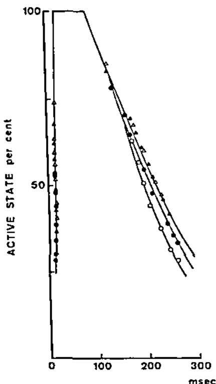


Fig. 2. A. Isometric myogram illustrating four partially fused twitches of single muscle fibre. B. photographically superimposed tension records illustrating the response to quick release carried out at different times during the first contraction cycle (only redevelopment of tension is shown). Contraction initiated at 2.2 μ sarcomere length; redevelopment of tension occurred at 2.1 μ. In the uppermost trace no quick release occurred but the fibre was allowed to shorten freely to 2.1 μ sarcomere spacing. Initial hump in the unreleased tension record due to inertia of lever during the shortening phase. Dashed lines indicate decay of active state during the first contraction cycle and the rise of activity initiated by the second stimulus. Horizontal line (top) represents maximal tetanic tension.

'active state' (Hill 1949) is used in the following to designate the capacity of the fibre to produce tension.

A. Procedure of the analysis. The decay phase of the active state was derived according to the quick release method devised by Ritchie (1954) for whole muscle. The rising phase of the active state was determined using the approach described in detail previously (Edman 1970). The procedure of the analysis is illustrated in Fig. 2. The fibre was carefully paced to produce a series of four incompletely fused isometric twitches every 2 min. The lever to which the fibre was attached was thereby fixed by a releasable catch (I, Fig. 1). The individual shocks in the stimulation train were applied at a frequency (3—6 per sec) selected so as to produce distinct peaks and troughs in the isometric myogram. At a pre-set time the lever was quickly released and the fibre was allowed to shorten 0.1 μ per sarcomere. Fig. 2 B shows a series of quick release recordings that were carried out during the first cycle superimposed on the same time base. The peaks and troughs of the isometric myogram both represent the tension output when the contractile unit is momentarily in equilibrium with the series elastic element. The tension recorded at these instances is therefore an adequate measure of the capacity of the contractile unit to produce tension, i.e. of the intensity of the active state at respective times after the stimulus (Ritchie 1954, Edman 1970). From the data illustrated in Fig. 2 B it was possible to derive the time course of the decay of the active state during the first cycle and the initial portion of the rising phase of the active state during the second cycle. Using a similar procedure the active state curves of the subsequent contraction cycles could be determined. The rising phase of the first contraction cycle was not accessible for analysis by means of the approach used.

Fig. 3 illustrates the rising and falling phases of the active state analyzed in the four different contraction cycles at 2.7 μ sarcomere length. It is seen, in confirmation of previous findings (Edman 1970), that the active state underwent a very rapid rise after the latency period. Only 5 msec were required for the activity to increase from 30 to 75 per cent of its maximal value. The results furthermore show that the same



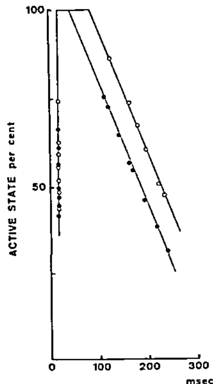


Fig 3 Fig 4

Fig 3 Time course of the active state analyzed through a series of four incompletely fused twitches ○ 1st ● 2nd ▲ 3rd △ 4th contraction cycles Sarcomere length 2 7 μ Active state maximum represents full tetanic tension

Fig 4 Rising and falling phases of the active state determined at 2 1 μ (filled circles) and 2 7 μ (open circles) sarcomere lengths in the same fibre Analysis carried out during 4th cycle of a series of four incompletely fused isometric twitches Active state maximum (maximal tetanic tension) normalized to 100 per cent at both lengths

steep rise of the active state existed in cycles 2—4 The decay of activity on the other hand became somewhat slower by restimulation of the fibre This effect was discernible between the first second and third contraction cycles whilst virtually identical curves were obtained during the third and fourth twitch periods In the experiment shown in Fig 3 the duration of the active state measured at 50 per cent of maximal activity was 175 msec during the first cycle and increased to 190 and 205 msec respectively during the second and third twitches of the series

B The kinetics of the active state in relation to sarcomere length The time course of the active state was determined at two or more different sarcomere lengths within the range 2 80—1 70 μ in the same fibre The analysis was carried out during the fourth cycle of an incompletely fused tetanus using the experimental approach described in the previous section When sarcomere lengths shorter than 2 1 μ wer

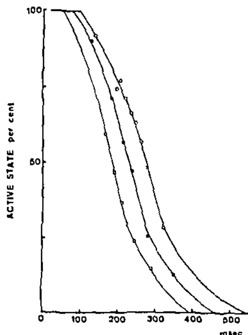
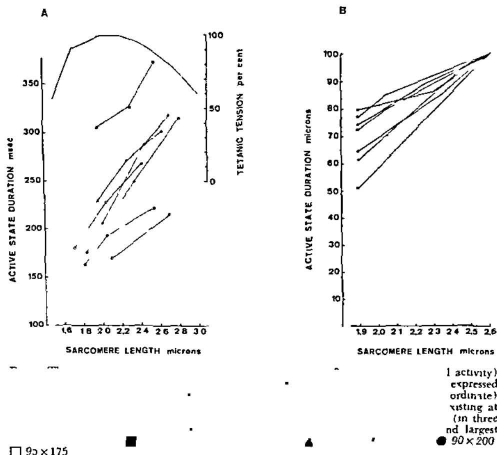


Fig. 5. Decay phase of the active state analysed at three different sarcomere lengths in the same fibre. ○ 2.6 μ; ● 2.3 μ; □ 1.95 μ. Analysis carried out during 4th cycle of a series of four incompletely fused isometric twitches. Active state maximum (maximal tetanic tension) normalized to 100 per cent at all three lengths.

studied, the contraction was initiated at 2.2 μ and the fibre was allowed to shorten against a small (4–5 dynes) load to a pre-set length. The quick release analysis (for determination of the rising and falling phases of the active state) was then carried out from the pre-shortened length in the same way as described in section A. The same amount of release (0.1 μ/sarcomere) was used at all lengths studied.

The results of two experiments are illustrated in Fig. 4 and Fig. 5. The experimental data shown in these diagrams have been expressed as percentage of the maximal tetanic output at the different lengths considered. The rate of rise of the active state was found to be independent of the sarcomere length. This is evident from Fig. 4 which illustrates a complete analysis of the active state at 2.7 μ and 2.1 μ sarcomere lengths. As can be seen the steepness of the initial phase was the same at both lengths. On the other hand, as is demonstrated in Fig. 4 and Fig. 5, the duration of the active state was steadily decreased by reduction in sarcomere length. It should be noted that the slope of the decay phase was not markedly changed; the curve was shifted in a nearly parallel fashion along the time axis. This finding suggests that the active state started to decay at an earlier time after the stimulus at the shorter length. The speed of decay, i.e. percental decline in activity per unit of time, was not markedly affected by changing the fibre length.

Fig. 6 summarizes the results of 7 expts. similar to those described in Fig. 4 and 5. Here the duration of the active state, measured at 50 per cent of maximal activity, has been plotted against the sarcomere length. Fig. 6 A also includes, for comparison, the curve correlating tetanic tension with sarcomere length. It can be seen that the



duration of the active state differed considerably from one fibre to another (also see Edman *et al* 1966) For instance at 2 1 μ sarcomere length the duration of activity ranged between 170 and 320 msec Attempts were made to find out whether this inherent difference in duration of active state between fibres somehow corresponded to differences in fibre thickness No such correlation (*cf* legends of Fig 6) was found however The data presented in Fig 6 A show in accordance with the findings described in Fig 4 and Fig 5 that for each individual fibre a nearly linear relationship existed between active state duration and sarcomere length However the dependence on length of the active state duration varied quantitatively from fibre to fibre This is further illustrated in Fig 6 B in which the duration of activity existing at 2 60 μ sarcomere spacing has been normalized to 100 per cent It can be seen that in the 7 expts demonstrated the duration of the active state measured at 1 9 μ sarcomere length varied between 51 and 80 (mean 69) per cent of the duration recorded at 2 6 μ

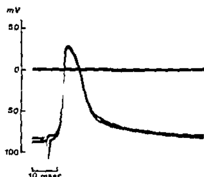


Fig 7 Superimposed oscilloscope traces illustrating almost identical shape of two action potentials recorded at 2 9 μ and 2 35 μ sarcomere lengths in the same fibre

The results would seem to make clear that the duration of the active state was not related to the tension output of the fibre This is evident from the fact that the active state was steadily abbreviated by reduction in length of the sarcomeres, irrespective of whether the isometric tension increased (shortening from 2 8 μ to 2 2 μ), remained constant (shortening from 2 2 μ to 2 0 μ) or was decreased (shortening *below 2 0 μ sarcomere length*) in consequence of the length change (*Fig 6 A*) These findings would seem to preclude that tension development, and hence the degree of interaction between the A and I filaments, exerts any significant feedback control upon the duration of the active state (Hill 1964)

Experiments were conducted to find out whether the length dependence of the active state as described above might be attributed to a change in the duration of the action potential (*cf* Edman *et al* 1966) Intracellular recordings of the action potential were made at different sarcomere lengths, within the range 2 9—2 1 μ using the same fibre Fig 7 shows the results of an experiment in which the action potential was recorded at 2 9 μ and 2 35 μ sarcomere lengths As can be seen almost identical recordings of the action potential were obtained at the two sarcomere lengths It should be noted for comparison that the mechanical activity was abbreviated by approximately 30 per cent by a similar change in sarcomere length (Fig 6) These findings indicate that the length dependence of the active state is not mediated by a change in the action potential mechanism

C Depression of the active state caused by active shortening of the contractile system The following experiments were designed to show whether active shortening of the contractile system affects the kinetics of the active state The analysis of the fibres capacity to produce tension was carried out at a given sarcomere length as described before but the amount of shortening of the sarcomeres after the quick release was varied Fig 8 illustrates the results of a typical experiment The full line (closed circles) represents the active state curve determined at 2 25 μ sarcomere length using the ordinary small release of 0 1 μ per sarcomere This amount of release was just sufficient to produce a complete drop in tension at the peak of the isometric twitch The open circles indicate the redeveloped peak twitch tension at 2 25 μ sarcomere spacing after a release of 0 25 μ per sarcomere The time intervals during which this larger shortening occurred are also indicated in Fig 8 The

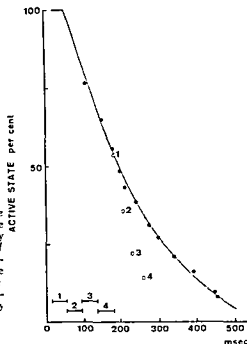


Fig 8 Reduction in tension development capability caused by active shortening Full line (closed circles) indicates decay of the active state at 2 25 μ sarcomere spacing analyzed by means of standard amount of release, 0 1 μ/sarcomere Open circles indicate peaks of redeveloped twitch tension at 2 25 μ sarcomere spacing after a release of 0 25 μ/sarcomere Time intervals during which the 0 25 μ/sarcomere release occurred are marked out by numbered horizontal bars

findings accord with results described previously in studies on whole skeletal muscle of the frog (Jewell and Wilkie 1960) It is clear that by allowing a bigger movement of the fibre the capacity of the contractile system to produce tension was diminished The effect was relatively small when the movement occurred while the active state was maximal, *i e* before 50 msec after the stimulus in the experiment of Fig 8, but became more and more pronounced when the shortening took place at a later time during the activity period

The nature of the movement effect is further elucidated in Fig 9 Here is illustrated the time course of the redevelopment of isometric tension starting at two selected times after the stimulus, the full lines after a release of 0 1 μ per sarcomere, the interrupted lines after a release of 0 25 μ per sarcomere The redevelopment of tension occurred at 2 25 μ sarcomere length in both cases The myogram of an unreleased twitch at 2 25 μ sarcomere length (top trace) is also shown It can be seen that both rate of tension development and twitch amplitude were reduced when a larger movement preceded The duration of of the twitch response however was not decreased [1] It can be inferred from these results that active shortening has a depressant effect upon the contractile process which persists throughout the rest of the activity period

presented in Fig 6

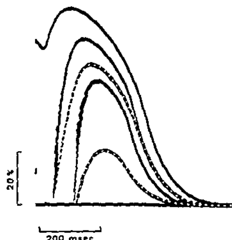


Fig 9 Isometric myograms illustrating redevelopment of tension (at 2 25 μ sarcomere length) at two different times after the stimulus Amount of release full lines 0 1 μ/sarcomere, interrupted lines 0 25 μ/sarcomere Uppermost myogram without release Note that although tension production is reduced after the larger movement the total duration of the twitch is not diminished Same experiment as in Fig 8 Vertical scale per cent of maximal tetanic tension

The total duration of the active state is apparently little affected The main effect would seem to be a decrease in the intensity of the active state as indicated by the relatively uniform reduction in tension output at different times after the isotonic phase It is of interest to note that the effect produced by a given amount of movement varies with the time at which the movement occurs during the contraction cycle The implication of this finding will be further discussed below

Discussion

The rising phase of the active state

The analytical approach used has enabled a detailed study of the rising phase of the active state in single muscle fibres that were stimulated to produce an incompletely fused tetanus The results have shown that only 5 msec are required (1—2° C) for the activity to rise from 30 to 75 per cent of its maximal value This means by extrapolation from the experimental data that the entire rising phase of the active state would be accomplished within a time interval of no more than 8—10 msec (from the end of the latency period) under the conditions studied here The development of the active state is thus considerably more rapid than might be inferred from previous experiments on whole muscle As demonstrated in studies on sartorius muscles from the frog (Hill 1949 Close 1962) and toad (Hill 1949) approximately 25 msec are required after the latency period before maximal active state (recorded as tension) is attained There is no definite explanation of this discrepancy in results It is reasonable to assume however as pointed out by Hill (1951) that the rate of rise of the active state is underestimated by analysis on the whole muscle due to the complex nature of the mechanical response from a large population of fibres For instance, differences in propagation velocity between individual fibres will cause an apparently longer rise time of the activity in the whole muscle as certain fibres will start later than others (unless massive stimulation is used The same kind of error is

introduced by differences in duration of the latency period between different fibres The present results have substantiated the assumption made by Hill (1951) however that the change in state from rest to full activity is not completely abrupt even on fibre level The initial portion of the rising phase has a definite slope corresponding to an increment in activity of approximately 10 per cent of P_{max} per msec

It should be pointed out that the rising phase of the active state also includes the time required for the mechanical activity to spread from the periphery of the fibre into its centre As demonstrated by Gonzalez Serratos (1966) the inward spread of activation occurs at a speed of 30 mm per sec at 5° C This means that for a fibre of ordinary thickness (approximately 100 μ) the central parts are being activated about 1 5 msec later than the peripheral layers The rise time of the active state considered at the level of the individual myofibril is thus 1—2 msec shorter than indicated by the output recorded from the fibre as a whole

The analysis of the active state provides relevant information for evaluation of the kinetics of the excitation contraction coupling (*e g* Edman *et al* 1966 Taylor 1969 Sandow 1970) It is now generally agreed that calcium functions as an activator of the contractile process in muscle According to the most accepted view calcium after being released from its storage site in the sarcoplasmic reticulum is assumed to combine with the troponin tropomyosin complex of the I filament thereby cancelling the inhibition of interaction between the actin and myosin components that otherwise exists (*e g* Ebashi and Endo 1968 Fuchs and Briggs 1968) On this basis then the active state parameter may be considered a useful index of the concentration of protein bound calcium in the myofibrillar space The present results would thus seem to indicate that the main portion of the calcium release including the transport of calcium into the myofibrils occurs within a time interval of 20 msec after the stimulus at the temperature used It is furthermore of interest to note that the slope of the initial phase of the active state is not markedly dependent on the sarcomere length This suggests that the rate of release of activator calcium from the storage places is not affected by changes in fibre length This piece of information will be used in the following section (Fig 10) in discussing the length dependence of the decay phase of the active state

The decay phase of the active state

Dependence on sarcomere length A reduction in sarcomere length within the range investigated in these experiments (1 7—2 8 μ) is associated with a decrease in duration of the active state This effect is largely attributable to the fact that the active state starts to decay earlier at a shorter length the speed of decay being virtually the same at all lengths concerned The observed changes of the active state curve are not related to the tension output of the fibre The duration of the activity is thus steadily abbreviated by decreasing the sarcomere length irrespective of how the tetanic output is affected by the length change This finding would seem to rule out that the amount of overlap and hence the degree of interaction between the A and I filaments plays any significant part in determining the duration of the active state

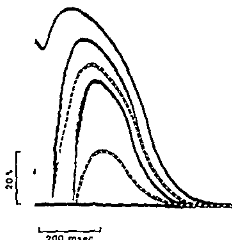


Fig. 9. Isometric myograms illustrating redevelopment of tension (at 2.25 μ sarcomere length) at two different times after the stimulus. Amount of release: full lines 0.1 μ/sarcomere, interrupted lines 0.25 μ/sarcomere. Uppermost myogram without release. Note that although tension production is reduced after the larger movement, the total duration of the twitch is not diminished. Same experiment as in Fig. 8. Vertical scale: per cent of maximal tetanic tension.

The total duration of the active state is apparently little affected. The main effect would seem to be a decrease in the intensity of the active state as indicated by the relatively uniform reduction in tension output at different times after the isotonic phase. It is of interest to note that the effect produced by a given amount of movement varies with the time at which the movement occurs during the contraction cycle. The implication of this finding will be further discussed below.

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that shortening somehow reduces the effective time of release of the activator (*cf* Adrian *et al* 1969) in spite of the fact that the duration of the action potential is unchanged The steep rise of the active state suggests that even a very small abbreviation of the activator release will cause a relatively large decrease in the total amount of activator set free

An observation probably related to the present results has recently been reported by Taylor and Rudel (1970) When a muscle fibre that is stimulated tetanically is allowed to shorten to a sarcomere length of approximately 1 6 μ the central myofibrils exhibit a wavy appearance suggesting that they are inadequately activated at this length The possibility exists that the centre of a fibre is not completely activated even at a stretched sarcomere length *i e* there may be an activity gradient from the periphery of the fibre towards the centre under all conditions On this basis the mechanism proposed in Fig 10 might also cover the finding described by Taylor and Rudel If the quantity of activator calcium that is released in each individual layer of the fibre is reduced by shortening a point may eventually be reached where the central myofibrils are not activated at all by membrane depolarization

Influence of movement The analysis has shown that movement *per se* affects the course of the active state independent of the change produced by altering the sarcomere length a period of free shortening interposed during an isometric twitch reduces the fibre s ability to produce tension without causing any substantial change in the total duration of the activity The fibre behaves as if a fraction of the contractile sites were uncoupled during the isotonic phase and that these sites did not come into function again until the fibre has been stimulated anew It is of interest to note that the decrease in tension output caused by active shortening is related to the degree of activity in the fibre when the movement occurs the effect evoked by a given amount of movement is thus insignificant as long as the active state is maximal but it becomes more and more pronounced the later the movement occurs during the decay phase of the active state This finding may be an indication (see below) that the depressant effect is somehow dependent on how much activator calcium is available at the contractile sites Evidence presented by Brady (1966 1968) would seem to indicate that a similar deterioration of the muscle s capacity to produce tension also occurs in response to active shortening of the mammalian myocardium

One intriguing possibility emerging from the present results would be that activator calcium is mobilized from the contractile proteins when the A and I filaments slide along each other during activity The fraction of calcium set free in this way may be quickly eliminated from the myofibrillar space by action of the pump mechanism in the sarcoplasmic reticulum This would lead to a reduction in the intensity of the active state provided the concentration of bound calcium is reduced below the level of mechanical saturation There is so far no direct experimental support for the existence of such a mechanism The idea of a mobilization of calcium is attractive however as it would seem to provide a reasonable explanation of the finding that the decrease in intensity of the active state becomes larger when the movement occurs after the activity has started to decay

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The Effect of Cooling on Liver Function in Cats

By

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Abstract

LARSEN, J A *The effect of cooling on liver function in cats* Acta physiol scand 1971 *81* 197—207

Liver function was followed by the elimination rate of glycerol and ethanol and the hepatic uptake of Indocyanine Green It was demonstrated that a 1° to 2° C reduction of the body temperature or cooling of the portal vein reduced the elimination rate of glycerol and ethanol, as well as the hepatic uptake of Indocyanine Green, about 40 per cent The experimental findings may be explained by restricted distribution of blood flow in the liver and a corresponding reduction of the functional capacity

The hepatic circulation has been the subject of intensive study in the last three decades Interest has mainly been focused on total hepatic blood flow and its regulation whereas the intrahepatic distribution of blood flow has recieved relatively little attention Several studies indicate that various intrahepatic vascular pathways may exist, but the results from these studies are very conflicting and at present the functional importance of distribution changes of liver blood flow is obscure (Bradley 1963, Brauer 1963)

The present article describes experiments on cats in which moderate hypothermia initiates marked changes in liver function, which to some degree can be quantitated and which may be explained by restricted distribution of liver blood flow and a corresponding reduction of functional liver tissue

Methods

The cats were anesthetized with chloralose (50 mg/kg) and an initial dose of 30 mg Nembutal They were kept in huge cages which allowed a high degree of physical activity and were given a standard diet consisting of boiled fish and milk 12 hrs prior to the experiment food was withdrawn but the cats had free access to water A femoral artery was cannulated and used for blood sampling and blood pressure recording Mean blood pressure recorded on a mercury manometer pulse rate and respiration were followed through-out the experiment A femoral vein was used for infusion Catheterization of a liver vein was performed via the external jugular vein and right heart The liver was exposed by a midline incision in the abdomen and the catheter was manually guided into a liver lobe

Temperature regulation and cooling In the control period the body temperature was kept constant between 38 0° and 38 5° C by heating Cooling was generally accomplished by surface cooling air being blown over the chest which was wetted with an alcohol solution In this w
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cold water In some experiments the portal vein was cooled locally by perfusing a plastic cap placed around the vein just before its entrance to the liver

Administration of glycerol and ethanol and calculation of elimination rate Liver function was followed by determination of the elimination rate of either glycerol or ethanol which are almost exclusively eliminated in the liver The elimination rate was measured at concentrations where the elimination capacity of the liver is saturated, thus being independent of concentration and within wide limits, of blood flow also (Larsen 1963 a b) A priming dose of glycerol or eth ... ion to about 7—10 mM ... owed by continuous infusic ... t a rate of 0 10—0 16 n ... weight so as to be approximately equal to the elimination rate at saturation One hour was allowed for equilibra ...

disappearing from the solvent space (68 per cent of the body weight) per unit time was calculated from the slope of the line which graphically best fitted the experimental points The elimination rate was then calculated by correcting the amount infused per minute by the amount retained in or disappearing from the solvent space (Larsen 1963 a b) In some experiments the hepatic elimination rate of glycerol and ethanol was calculated by multiplying the arterio-hepatic venous difference of these substances by the liver blood flow as determined by means of Indocyanine Green (ICG)

Administration of ICG Liver function was also followed by infusion of ICG This substance is exclusively eliminated in the liver and in contrast to glycerol and ethanol the hepatic ... 1961) The arterial concentration ... ed by continuous infusion at a rate ... ially resulted in a horizontal time ... (FHBF) was then calculated after

Analytical procedures Glycerol and ethanol were determined enzymatically as described in detail elsewhere (Larsen 1963a b) Free fatty acids were determined as described by Dole and Meinertz (1960) ICG was determined spectrophotometrically with a special correction for blank density (Nielsen 1963) Oxygen uptake was measured by connecting the tracheotomized cat to a closed system containing recirculating oxygen Determination of arterial O_2 and CO_2 tensions as well as pH were performed by means of standard apparatus manufactured by Radiometer Copenhagen

Statistical procedures Students t test for paired observations was used for calculation of probability of difference (p)

Results

Effect of cooling on the elimination rate of glycerol The results from a series of experiments in which the effect of cooling on the elimination rate of glycerol was tested are listed in Table I One experiment in which cooling was followed by rewarming is illustrated in Fig 1 It will be noticed that after a temperature fall of about 1° C there is a sudden decrease (33 per cent) in the elimination rate A further drop in temperature is without significant effect on the elimination rate When rewarmed there is a sudden increase in elimination rate when the temperature reaches the level at which the decrease was initiated The elimination rate is now 61 μmoles/kg/min as compared to 54 μmoles/kg/min in the first period Cat 7 and

TABLE I The results from experiments with continuous infusion of glycerol (— indicates technical failure)

Cat no	Elimination of glycerol (μmoles/kg/min)		Decrease in elimination per cent	Mean blood pressure mm Hg		Pa_{O_2} mm Hg		Pa_{CO_2} mm Hg		pH (arterial)	
	A	B		A	B	A	B	A	B	A	B
1	54	36	33	140	125	90	100	37.7	37.9	7.34	7.34
2	43	19	46	90	75	83	80	48.9	57.0	7.33	7.27
3	42	21	50	120	80	83	95	42.5	45.0	7.28	7.26
4	53	33	38	85	100	—	—	—	—	—	—
5	37	25	32	155	125	—	—	—	—	—	—
6	53	29	45	110	115	80	—	36.6	—	7.30	7.33
7	60	40	33	105	110	83	90	38.0	36.0	7.32	7.33
8	45	38	16	135	135	79	82	47.1	48.0	7.31	7.31
9	50	35	30	115	110	65	70	44.6	42.5	7.29	7.31
10	27	15	44	85	60	85	90	35.1	33.8	7.31	7.32
Mean	46	29	38	114	104	81	87	41.3	42.9	7.31	7.31
S E	3.1	2.7	3.6	7.6	7.7	2.6	3.8	1.8	3.0	0.01	0.01
p	< 0.001			$0.05<p<0.10$		$0.020<p<0.025$		$0.5<p<0.6$		$0.95<p<0.90$	

A control period
B cooling period

9 were also rewarmed and a corresponding increase in the elimination rate to 50 and 46 μmoles/kg/min, respectively, was observed In these experiments the cats were cooled to 36.5° C and in every experiment cooling decreased the elimination rate of glycerol the mean decrease being 38 per cent The change in elimination rate was *not accompanied by significant changes in* mean blood pressure, blood gas tensions or pH The plasma concentration of free fatty acids was followed in cats 2 3 and 4 It was found that the concentration of free fatty acids remained constant in the experimental period In cat 4 noradrenaline was infused at the end of the experiment and this caused an immediate increase in plasma concentration of both glycerol and free fatty acids The effect of cooling was also seen when the activity of striated muscles was abolished by intravenous administration of tubocurarin (3 mg/ml) (cat 5) The effect of intra abdominal cooling was comparable to the effect of surface cooling (cat 6) In cat 7 the hepatic plexus was cut and a 2 % solution of Lidocain was applicated locally without effect on the reaction to cooling In cats 8 9 and 10 the body temperature was kept constant while the portal vein was locally cooled *In these experiments also cooling caused a decrease in elimination rate of glycerol*

Renal excretion was measured in 2 expts (2 3) and was found to be reduced from 10 to 7 and 15 to 7 μmoles/kg/min by cooling In these 2 expts the elimination rate has been corrected for renal excretion In 3 expts (1 4 7) renal excretion was prevented by ligation of the ureters In the rest of the experiments the elimination rate includes renal excretion Some experiments not listed here showed the following Both carotid arteries were cooled without any effect on the elimination rate of glycerol Cooling did not effect the elimination rate of glycerol in eviscerated animals

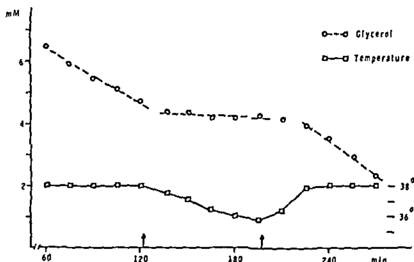


Fig 1 *The elimination curve of glycerol during continuous infusion of glycerol* *The arrows* indicate time of cooling and heating Ordinate glycerol concentration in arterial blood Abscissa time (cat no 1)

The effect of alfa- and betareceptor blocking agents was examined in 2 expts In one propanolol (1 mg/kg) and in the other, phenoxybenzamine (15 mg/kg) given i v at the beginning of the experiment did not abolish the effect of cooling

Other induced changes in the elimination curve of glycerol It was thought of interest to compare the cooling-induced changes in the elimination curve of glycerol with those seen by suddenly changing the infusion rate or by ligation of a liver lobe The results are illustrated in Fig 2 Curve I shows an experiment in which the cat

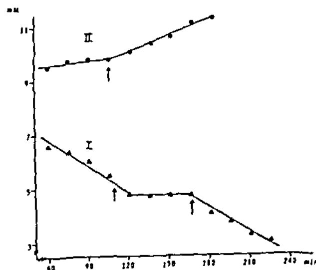


Fig 2 The elimination curve of glycerol during continuous infusion of glycerol *Curve I illustrates the effect of cooling to 36 5° C (first arrow) and then changing of infusion rate (second arrow)* Curve II illustrates the effect of ligation of a liver lobe (at arrow) *Ordinate* glycerol concentration in arterial blood Abscissa time

TABLE II The results from experiments with continuous infusion of ethanol

Cat no	Elimination of ethanol (μmoles kg/min)		Decrease in elimination per cent
	A	B	
11	41	21	49
12	38	23	39
13	30	16	47
14	33	21	36
15	42	21	50
16	46	35	24
17	36	17	53
18	43	33	23
Mean	39	23	40
S E	1 9	2 5	4 1
P	< 0 001		

A = control period
B = cool ng period

was cooled to 36 5° C After about 10 min of cooling the elimination rate was suddenly and sharply reduced (from 53 to 27 μmoles/kg/min) 45 min later the infusion rate was decreased by 33 per cent This change was immediately followed by an apparent increase in elimination rate (from 27 to 37 μmoles/kg/min) Curve II illustrates an experiment in which a liver lobe was suddenly ligated This was accomplished by placing a loose ligature at the root of a liver lobe at the beginning of the experiment The ligature was then left undisturbed until the moment of tightening It appears that ligation is followed by an immediate decrease in elimination rate from 31 to 19 μmoles/kg/min (38 per cent), which was comparable to the size of the ligated lobe

Effect of cooling on the elimination rate of ethanol The effect of cooling on the elimination rate of ethanol was examined in a series of experiments the results of which are listed in Table II In these experiments the temperature was lowered from 38 5° C to 36 5° C by surface cooling The change in elimination curve upon cooling was identical to the findings in the glycerol experiments as illustrated in Fig 1 It will be noticed that the change in elimination rate is of the same order of magnitude as found with glycerol In some experiments not listed here ethanol was infused together with glycerol In these experiments the elimination of glycerol was decreased by about 40 per cent by cooling whereas the elimination rate of ethanol was reduced by a smaller degree namely about 30 per cent At the concentration levels of ethanol found in the present experiments renal excretion of ethanol is negligible

Effect of cooling on the elimination rate of glycerol and oxygen consumption In a series of experiments the elimination rate of glycerol was measured together with oxygen uptake In these experiments the ureters were ligated The results are listed in Table III The fall in body temperature is accompanied by a decrease in oxygen uptake of about 8 per cent per degree centigrade corresponding to a Q_{10}-value of

TABLE III The results from experiments in which the elimination rate of glycerol and oxygen uptake were measured at different body temperatures For comparison the results are given as percentage change

Cat no	Change in body temp	Decrease in glycerol elimination (per cent)	Decrease in oxygen uptake (per cent)
19	38 5—36 0	49	17
20	38 5—36 5	26	13
21	38 5—36 5	59	16
22	38 5—36 5	30	17
23	38 5—37 5	0	9
	37 5—36 5	45	7
24	38 5—37 5	0	—
	37 5—36 5	33	—

about 2 In 2 expts (cats 23 and 24) the body temperature was changed stepwise, one degree at a time The results reveal that lowering the temperature one degree from normal does not change the elimination rate of glycerol, whereas the oxygen consumption decreases according to a Q_{10}-value of about 2 A further decrease in temperature of one degree is accompanied by a marked fall in the elimination rate of glycerol whereas the fall in oxygen consumption is almost unchanged

The effect of cooling on the hepatic uptake of Indocyanine Green (ICG) and on Estimated Hepatic Blood Flow (EHBF)

In 9 experiments listed in Table IV glycerol or ethanol was infused together with ICG In these experiments a liver vein was cannulated and the arterial and hepatic venous concentrations of these substances were followed The ureters were ligated in the experiments where glycerol was infused

The effect of cooling on the elimination curves is illustrated in Fig 3 The arterial and hepatic venous concentrations of ICG were usually almost constant in the control period Upon cooling the arterial concentration of ICG increased in every experiment, and 45—60 min after cooling the arterial and hepatic venous concentrations leveled off at a higher level indicating that the amount taken up by the liver again equalled the amount infused After an initial decrease in the arterio-hepatic venous difference the difference in most experiments approached the precooling value, indicating that the EHBF was unchanged

For further evaluation of the liver function the extraction ratio for ICG (arterio-hepatic venous difference (mg/l)/arterial plasma concentration at equilibrium (mg/l)) and plasma clearance (hepatic uptake (μg/kg/min)/arterial plasma concentration at equilibrium (μg/ml)) were calculated Both parameters were found to decrease about 40 per cent upon cooling corresponding to the increase in arterial concentration of ICG

The hepatic elimination of glycerol and ethanol in the control period calculated from the arterio-hepatic venous difference of these substances and EHBF was ex-

TABLE IV The results from experiments in which the elimination rate of glycerol and ethanol and the hepatic uptake of Indocyanine Green were measured at normal body temperature and after reduction of body temperature to 36 5° C The hepatic elimination rate of glycerol and ethanol was calculated from the arterio-hepatic venous difference and EHBF The numbers in brackets represent the elimination rate as determined from the elimination curve

Cat no	Hepatic elimination (μmoles kg/min) glycerol		Extraction ratio (per cent) ICG		Plasma Clearance (ml/kg/min) ICG		Increase in arterial concentration (per cent) ICG	Estimated hepatic blood flow (ml kg/min)	
	A	B	A	B	A	B		A	B
25	52 (34)	31 (24)	22	20	10 2	6 9	46	65	49
26	20 (33)	11 (19)	38	16	7 7	5 7	31	31	53
27	12 (31)	9 (18)	57	45	6 2	4 2	40	16	16
28	13 (31)	4 (7)	44	26	7 5	4 6	61	30	29
29	19 (19)	14 (11)	31	17	7 5	3 5	100	35	30
30	22 (26)	16 (11)	30	12	6 0	2 3	142	30	30
Mean	23 (29)	14 (15)	37	23	7 5	4 5	70	35	35
S E	6 0	3 8	5 0	4 9	0 6	0 7	17 5	6 6	5 6
p	0 02 < p < 0 025		0 005 < 0 < 0 01		p < 0 001				
	Ethanol								
31	23 (31)	13 (9)	23	22	3 4	2 7	47	55	44
32	23 (33)	21 (18)	25	21	6 4	4 0	55	38	28
33	23 (36)	12 (26)	22	14	8 5	6 1	42	54	59
Mean	23 (33)	15 (18)	23	19	6 1	4 3	48	49	44
S E	0 0	2 8	0 9	2 5	1 5	1 0	3 8	5 5	9 0

A = control period
B = cooling period

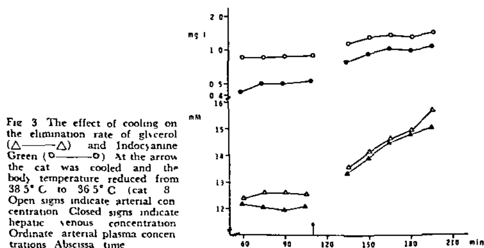


Fig 3 The effect of cooling on the elimination rate of glycerol (△——△) and Indocyanine Green (○——○) At the arrow the cat was cooled and the body temperature reduced from 38 5° C to 36 5° C (cat 8 Open signs indicate arterial concentration Closed signs indicate hepatic venous concentration Ordinate arterial plasma concentrations Abscissa time

TABLE III *The results from experiments in which the elimination rate of glycerol and oxygen uptake were measured at different body temperatures For comparison the results are given as percentage change*

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23	38 5—37 5	0	9
	37 5—36 5	45	7
24	38 5—37 5	0	—
	37 5—36 5	33	—

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The hepatic elimination of glycerol and ethanol in the control period calculated from the arterio-hepatic venous difference of these substances and EHBF was ex

tion in temperature It therefore seems reasonable to exclude a Q_{10} effect of tempera ture as an explanation for the effect of cooling

As pointed out previously the elimination rate of glycerol and ethanol might be influenced by a severe reduction of liver blood flow and therefore the flow was measured in a series of experiments by means of continuous infusion of ICG The experiments revealed that total EHBF was not changed significantly by cooling The calculated flow was of the order of magnitude as previously found in cats by a dif ferent method (Larsen 1963 a b) and was also in accordance with flow values found in dogs (Fischer 1963) These experiments confirmed that the hepatic elimina tion rate of glycerol and ethanol is decreased by cooling In the control period the hepatic elimination rate as calculated from the arterio-hepatic venous difference and EHBF was in all cases except one smaller than the elimination rate calculated from the elimination curve This descrepancy may to some extent be explained by the existance of an extrahepatic elimination of glycerol and ethanol which amounts to 10—20 per cent of the total elimination (Larsen 1959 1963 a) The calculation of the elimination rate from the elimination curve is also subject to a certain systematic error caused by the fixed calculation of solvent space Both the extrahepatic elimina tion rate and the solvent space may however be taken as constants in the single ex periment and they are therefore not considered as major errors in the interpretation of the results It is also possible that the descrepancy may be caused by local changes in blood flow and elimination capacity caused by the catheter

The increase in arterial concentration of ICG upon cooling is most probably caused by a reduction in hepatic uptake which is then compensated by an increase in concentration This reaction is in accordance with the well established fact that the hepatic uptake of ICG is dependent on concentration The raise in concentration in combination with the unchanged or slightly changed EHBF results in a reduc tion in both plasma clearance and in the extraction ratio of ICG of about 40 per cent

The observed changes in hepatic function may be explained by assuming that cooling causes restricted distribution of blood flow within the liver with reduction of the functional capacity of the liver tissue A sudden and reversible change in vascular patterns could explain the all or non reaction to cooling It might also ex plain why the hepatic elimination rate of three substances with different metabolic pathways is reduced to the same great extent It should be stressed that although the mean decrease in hepatic clearance of ICG and the hepatic elimination of glycerol or ethanol is of the same magnitude the correlation between these parame ters is poor However it is difficult to imagine that restricted distribution of flow should mean complete closure of certain parts of the liver It rather means local changes in tissue perfusion rates with a decrease in the restricted parts of the liver and with constant total liver blood flow a corresponding increase in other parts of the liver It would be expected that such changes may effect the hepatic uptake of ICG in a different way than the hepatic elimination of glycerol or ethanol

The existense of uneven perfusion of liver tissue has been under dispute

years This subject has been thoroughly reviewed by Brauer (1963) and Bradley (1963), but a few papers especially pertinent to the present discussion will be referred to here Based on the anatomical arrangement of the hepatic vessels Rappaport (1963) and Elias and Sherrick (1969) claim that the very structure of the hepatic parenchyma suggests that the blood does not simply flow through pre established channels but must be shifted along different vascular pathways by a steering mechanism This view is supported by several studies in which in vivo observations of the sinusoidal blood flow showed intermittency of flow (Deysach 1941 Knisely, Block and Warner 1948 Seneviratne 1950, Wakim and Mann 1953, Bloch 1955) Intermittency of sinusoidal flow has been denied by others e g Maegraith (1958) or has been reduced to take place only in the so called ladder sinusoids which connect major sinusoidal channels (Nakata Leong and Brauer 1966) Recently McCuskey (1966) in an extensive study on frogs white rats mice and rabbits has demonstrated both intermittency of sinusoidal flow and the existance of sphincter activity of the sinusoids

Angiographic studies by Daniel and Prichard (1951 a b) revealed restricted intra hepatic blood flow in which the peripheral parts of the liver were more or less deprived of their blood supply and by means of heated thermocouple technique rhytmical fluctuations in the intrahepatic blood flow were demonstrated in man (Graf Graf and Rosell 1958)

In relation to the present experiments it is of interest to note that Seneviratne (1950) observed that cooling of the animals surface as well as local cooling of the liver was accompanied by contraction of the sinusoids which remained contracted as long as the cooling lasted On discontinuing the cooling the sinusoids rapidly returned to normal

The litterature contains relatively little and conflicting information regarding the effect of hypothermia on liver function and most experiments have been concerned with the effect of deep hypothermia (Brauer *et al* 1959) It appears that bile flow is especially sensitive to changes in temperature (Brauer *et al* 1959) and also the hepatic metabolism of glucose and fructose seems to be markedly influenced by cooling (Wynn 1954 1956 Bickford and Mottram 1960)

In conclusion the present experiments demonstrate that a slight fall in liver temperature elicits characteristic changes in liver function which may be explained by a restriction of the intrahepatic circulation *It should be stressed that the response* to cooling may be quite different in the unanesthetized animal On the other hand the observed changes in liver function may be induced at temperatures within the normal range of body temperature As a consequence local changes in metabolism and thereby temperature or vice versa may play a hitherto unknown role in the regulation of liver function More experimental data are needed before the observed effect of cooling can be satisfactorily explained However experiments in progress including angiography and studies of the reaction of bile flow to cooling so far support the proposed explanation for the effect of cooling on liver function

The technical assistance of Mrs J Justesen is gratefully acknowledged

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Heparin-Induced Increase of Diamine Oxidase in Lymph and Blood Plasma of Rat, Guinea-Pig and Rabbit

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Abstract

HANSSON R and H THYSELL *Heparin-induced increase of diamine oxidase in lymph and blood plasma of rat, guinea pig and rabbit* Acta physiol scand 1971 *81* 208—214

After i v injection of heparin (1 6 IU/g b w) there was a significant increase of the diamine oxidase (DAO) activity in lymph and blood plasma in rat guinea pig and rabbit The ratio between lymph and plasma DAO activity was higher in rat and rabbit than in guinea pig under basal conditions This difference was even more pronounced after heparin injection Withdrawal of mesenteric or thoracic duct lymph from the circulation of the animals was accompanied by a poor response to heparin of the plasma DAO activity in rat and rabbit whereas the response in the guinea pig seemed to be uninfluenced These results suggest that in analogy with earlier findings in man the contribution from lymph to the heparin induced DAO response in blood plasma may be significant in the rat and rabbit but not in the guinea pig

60 years ago it was shown that during anaphylactic shock in the dog there was a marked increase in the flow of thoracic duct lymph and that the lymph did not coagulate (Calvary 1909) Twenty years ago the basal level of histaminolytic activity of canine thoracic duct lymph was reported to be about 30 times higher than that of blood plasma (Carlsten *et al* 1949) and a similar ratio was found in cats and rabbits (Carlsten 1950)

The relation between anaphylactic shock and increased histaminase activity in blood plasma of rabbit (Rose and Leger 1952) white rat (Code *et al* 1961) and guinea pig (Logan 1961 has been established A series of investigations on guinea pig anaphylaxis convincingly indicated heparin as the mediator of the histaminase liberation into blood plasma for references see Schmutzler 1966 1968 Giertz and Hahn 1969)

The diamine oxidase DAO *i e* histaminase diamine oxygen oxidoreductase (deaminating) EC 1 4 3 6 —activity in blood plasma is markedly increased in a dose-dependent fashion for a few hours after i v administration of heparin to man

(Tryding 1963) and all vertebrate species hitherto investigated (Hansson and Thysell 1968)

In man the basal DAO activity of thoracic duct lymph is several times higher than that of blood plasma, and after i v injection of heparin this enzyme activity may be as much as 200 times higher in lymph than in blood plasma (Hansson *et al* 1966 Dahlback *et al* 1968) It is thus, likely that the lymph may give a delayed but significant contribution of DAO activity to the blood plasma after heparin injection and possibly, also during anaphylactic shock In order to establish whether analogous conditions exist in other mammals the effect of i v injection of heparin upon the DAO activity in lymph and blood plasma was investigated in rat guinea pig and rabbit

Material and Methods

The experimental procedure with respect to drugs cannulation injections (jugular vein)

cisterna in two animals the cisterna was drained by intermittent suction into sampling vessels In the remaining 2 rats the lymphatics were kept intact only laparotomy and intestinal exteriorization was performed as sham operation (sh in Fig 1) For comparison four other white rats with intact abdomen were studied ("n in Fig 1)

Guinea pigs 11 guinea pigs of both sexes body wt 280—675 g Ether anesthesia Laparotomy and lymph drainage (see under rats) in 2 animals of both dose groups (*i e* 0 2 and 1 6 IU heparin/g), sham operation in 2 of the lower dose groups ("sh), no laparatomy in 2 of both dose groups (n), and in one animal no laparotomy but incision in the left supraclavicular region dissection for exposure of the thoracic duct ligature of the venous receptaculum from its venous contributing branches in order to form a cistern for collection of lymph

Rabbits Seventeen rabbits of both sexes body wt 1 63—3 08 kg Local anesthesia completed by embomalum (NFN) narcosis when laparotomy was to follow upon cannulation (Narkotal® Astra Sweden 3—5 ml 55 mg/ml) 5 rabbits (n) were only cannulated for injection of heparin (jugular vein) and sampling (carotid artery) 5 rabbits were sham operated (sh) and another 5 animals were similarly operated but as much lymph as possible was drained from the junction of the mesenteric lymphatic trunk with the cisterna chyli In all the experiments blood and lymph samples were collected in micro test tubes and the corpuscular elements were sedimented in the centrifuge The cell free plasma and lymph were then stored at −20° C until analysis

Finally from 2 animals lymph was withdrawn from the thoracic duct in or near its orifice in the venous angle One of the rabbits received only local anesthesia the other full narcosis with Narkotal and lymph was drained simultaneously from the mesenteric lymph vessel (Fig 1)

Results and Conclusions

Whereas the basal diamine oxidase activity of blood plasma was rather high mean 0 21 U/L) in the guinea pigs as compared to the other species (mean for rats 0 04 and rabbits 0 04 U/L) the reverse seemed to be true for mesenteric and in one guinea pig thoracic) lymph (means 0 12 against 1 3 and 1 9 U/L respectively)

In every individual animal an increase of the DAO activity occurred both in lymph and blood plasma after i v injection of heparin There was however a quantitative difference between the guinea pigs on one hand and rats and rabbits on the other (Fig 1)

In the two rats the initial DAO ratio between mesenteric lymph and blood plasma was rather high (80 and 13) and increased further (to 420 and 1900, respectively), *after i v injection of 1 6 IU heparin/g b w* Sham operation did not reduce the heparin-induced DAO response in blood plasma (Fig 1 RAT), whereas a rather poor DAO response was seen in blood plasma after withdrawal of lymph (about 130 and 150 mU of DAO in the collected lymph specimens)

After heparin injection, both after 0 2 and 1 6 IU/g b w , the diamine oxidase (DAO) activity in guinea-pig blood plasma showed a rapid increase to a level of 20 and 40 U/L, respectively The increase of this enzyme was slower in the lymph specimens resulting in a temporary fall in the ratio between lymph and blood plasma DAO activity *Later during the experiment, this ratio increased but did not exceed* 0 5 in the animals given 0 2 IU heparin and 1 2 in those given 1 6 IU heparin per g b w Sham operation or withdrawal of lymph had no overt effect on the blood plasma DAO *response as compared to guinea-pigs with their abdomen intact*

In general the DAO activity of blood plasma was highest in those rabbits, which were under local anesthesia and had their abdomen and lymphatic trunks intact (*Fig 1, shaded area,* n) and lowest in those under general anesthesia and lymph deprivation via laparotomy (Table I, in Fig 1, hatched area, the lymph drained rabbits is a mixed group with and without laparotomy and general anesthesia) The sham operated rabbits formed an intermediate group (Table I Fig 1, pepper-and-salt sh) The overlap is considerable but for the period of 20 to 60 (and 90) min after heparin injection the difference of the 3 mean values are statistically significant (Table I) The amount of DAO activity collected in the lymph specimens from each animal throughout the experiment, was estimated to about 0 2 U (range 0 06 to 0 4 U) The ratio between lymph and blood plasma DAO activity was about 80 (range 21 to 200 before and as a maximum value about 280 (range 130 to 640) 20 to 45 min after the heparin injection The maximum value of DAO activity in chylous lymph after heparin administration varied between 7 and 125 U/L In one

TABLE I Diamine oxidase activity U l in rabbit blood plasma after i v injection of heparin

Min after heparin injection	Local anesthesia and intact abdomen normal Rabbit no					General anesthesia Narkotal® Astra Sweden and sham operation sham' Rabbit no				
	1	2	3	4	5	1	2	3	4	5
20	181	1 61	696	2 39	413	254	316	485	159	202
30	573	1 79	2 16	5 31	1 03	272	593	760	722	330
45	1 20	2 09	3 64	6 94	3 07	453	1 40	720	383	230
60	1 54	2 01	4 27	7 13	3 38	453	2 28	1 11	266	294
90	1 64	1 20	3 98	7 09	2 79	1 04	2 25	1 08	230	388

If a lognormal distribution of the data is assumed (cr ref e g Oldham 1968) then the variances of the 3 groups—'normal sham and drained show no significant differences (Variance ratio or F test, F = 1.21 and 1 18 respectively critical value f r 10 % significance level = 4 11) More

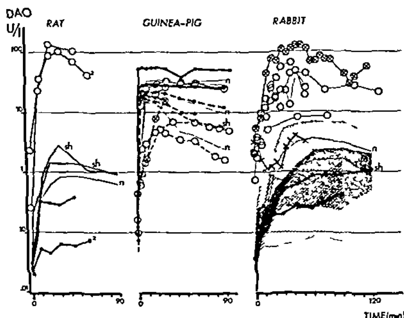


Fig 1 Diamine oxidase (DAO) activity in blood plasma and lymph of rat guinea pig and rabbit before and after i v heparin

n and grayish shade represent blood plasma DAO activity in animals with intact abdomen and lymph circulation "sh and pepper and salt shade represent sham operated animals bold lines (without letters) and hatched shade represent animals deprived of mesenteric (O) or thoracic duct(⊗) lymph or both by separate sampling (O and X RABBIT)

Outlines of shaded area represent range

Numerals (1 and 2 RAT) identify two animals

Interrupted lines (GUINEA PIG only) idicate 0 2 IU and solid lines 1 6 IU heparin per g b w

plus drain of mesenter c lymph
(dra ned) Rabb t no

1	2	3	4	5	6
055	112	161	077	133	154
062	221	144	094	103	134
067	464	165	077	094	169
086	678	103	068	0 82	125
072	—	—	053	083	—

over the mean values of the plasma DAO activity dur ng the observation period (70 to 60 and 20 to 90 min) d ffer from each other (t test) normal sham (005 > p > 001) and sham > drained (005 > p > 001)

In the two rats the initial DAO ratio between mesenteric lymph and blood plasma was rather high (80 and 13) and increased further (to 420 and 1900 respectively) after i.v. injection of 1.6 IU heparin/g b.w. Sham operation did not reduce the heparin induced DAO response in blood plasma (Fig. 1 RAT) whereas a rather poor DAO response was seen in blood plasma after withdrawal of lymph (about 130 and 150 mU of DAO in the collected lymph specimens).

After heparin injection both after 0.2 and 1.6 IU/g b.w. the diamine oxidase (DAO) activity in guinea pig blood plasma showed a rapid increase to a level of 20 and 40 U/L respectively. The increase of this enzyme was slower in the lymph specimens resulting in a temporary fall in the ratio between lymph and blood plasma DAO activity. Later during the experiment this ratio increased but did not exceed 0.5 in the animals given 0.2 IU heparin and 1.2 in those given 1.6 IU heparin per g b.w. Sham operation or withdrawal of lymph had no overt effect on the blood plasma DAO response as compared to guinea pigs with their abdomen intact.

In general the DAO activity of blood plasma was highest in those rabbits which were under local anesthesia and had their abdomen and lymphatic trunks intact (Fig. 1 shaded area n) and lowest in those under general anesthesia and lymph deprivation via laparotomy (Table I in Fig. 1 hatched area the lymph drained rabbits is a mixed group with and without laparotomy and general anesthesia). The sham operated rabbits formed an intermediate group (Table I Fig. 1 pepper and salt sh). The overlap is considerable but for the period of 20 to 60 (and 90) min after heparin injection the difference of the 3 mean values are statistically significant (Table I). The amount of DAO activity collected in the lymph specimens from each animal throughout the experiment was estimated to about 0.2 U (range 0.06 to 0.4 U). The ratio between lymph and blood plasma DAO activity was about 80 (range 21 to 200) before and as a maximum value about 280 (range 130 to 640) 20 to 45 min after the heparin injection. The maximum value of DAO activity in chylous lymph after heparin administration varied between 7 and 125 U/L. In one

TABLE I. Diamine oxidase activity (U/L) in rabbit blood plasma after i.v. injection of heparin

Min after heparin injection	Local anesthesia and intact abdomen (normal) Rabbit no					General anesthesia (Narkotal[R] Astra Sweden, and sham operation (sham)) Rabbit no				
	1	2	3	4	5	1	2	3	4	5
20	181	[illegible]	[illegible]	[illegible]	413	254	316	485	150	[illegible]
30	573	1 79	2 16	5 31	[illegible]	[illegible]	593	760	722	330
45	1 20	2 09	[illegible]	[illegible]	3 07	453	1 40	720	383	230
60	1 54	2 01	4 27	7 13	3 38	453	2 28	1 11	[illegible]	[illegible]
90	1 64	[illegible]	3 98	7 09	[illegible]	604	2 25	1 08	230	385

If a lognormal distribution of the data is assumed (for ref. e.g. Oldham 1968) then the variances of the 3 groups — normal, sham and drained — show no significant differences (Variance ratio or F test F = 1.21 and 1.18 respectively, critical value for 10 % significance level 4.11).

slower increase leads to the maximal value in the temporal course of the curve The lack of a two step escalation of blood plasma DAO activity after heparin has earlier been reported in man, especially when thoracic duct lymph was drained from the circulation (Dahlback *et al* 1968) In rats and rabbits similar effects were found when mesenteric lymph was withdrawn in the present investigation

The guinea pig reacts after i v injection of heparin with a rapid rise of the blood plasma DAO activity to an early and comparatively high peak value (after small doses of heparin, *e g* Schmutzler 1966, Hansson and Thysell 1968) or a plateau (after large doses of heparin) Our present findings do not suggest a substantial contribution of DAO from the guinea pig lymph to the systemic circulation and thus confirm earlier concepts (Schmutzler 1968), although even the guinea pig lymph DAO activity shows a significant increase after i v heparin The single DAO activity maximum in the guinea pig blood plasma may, consequently, be explained by a direct access of the enzyme to the blood and a negligible later contribution by the major lymphatics

Endogenous heparin is transferred to the extracellular space during anaphylactic shock as a consequence of mast cell degranulation In the dog 'heparin, once released into the tissue spaces of the liver, finds ready access to the blood by way of the thoracic duct (Riley 1964) In other species less convincing evidence of heparinaemia during anaphylactic shock was reported until a thorough study was performed on the guinea pig (for ref Gietz and Hahn 1969) In this species moreover heparin was considered to be the mediator of anaphylactic histaminase release In the literature, as it seems, corresponding studies on anaphylatic shock in other species are still lacking Fragmentary data exist, showing an increased DAO ('histaminase') activity in the blood plasma during anaphylactic shock in species, where a concomitant heparinaemia has not (yet) been demonstrated The i v administration of heparin does not necessarily mimic the heparin induced events during the anaphylactic shock the effect of endogenous heparin liberated into the tissues outside the systemic circulation remains to be studied The presence of DAO in the blood during anaphylactic shock in species without known heparinaemia is compatible with a DAO liberation possibly heparin induced and from the extravascular space Furthermore it may be worth while revaluating the role of lymph during the study of anaphylactic shock

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Acta physiol. scand 1971. 81. 215—224

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Ventilation and Heart-Rate Responses to Ramp-Function Changes in Work Load

By

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Abstract

Karlsson, H and O Wigertz *Ventilation and heart rate responses to ramp function changes in work load* Acta physiol scand 1971 *81* 215—224

Ventilation and heart rate responses were recorded continuously and simultaneously during supine leg exercise in [illegible]
starting or ending at [illegible]
The parameter T_L (' [illegible]
tionary condition lags [illegible]
were used the duratio [illegible]
For ventilation T_L was found to be largely independent of the slope of the ramp and [illegible]
to the two steepest ramps, T_L was negatively correlated to work capacity ($r = -0.968$ and -0.824)

In previous studies from this laboratory, deterministic input functions have been employed to find the dynamic characteristics of cardio respiratory responses to muscular exercise in man Thus sinusoidal (Wigertz 1970) and step (Broman and Wigertz 1970) changes in work load yielded results that could be described mathematically in the form of transfer functions, defining the dynamic input-output relationships between work load, on one hand and ventilation and heart rate on the other So far ramp inputs seem not to have been tested in man

The present investigation was carried out in order to examine the suitability of ramp testing for studying the dynamics of the ventilatory and circulatory adjustments to muscular exercise and furthermore to explore whether the dynamic properties might be influenced by altering the slope of the work load ramp As in the previous studies the experimental data were obtained from male athletes performing supine leg exercise on a cycle ergometer Some of the results have been briefly reported (Wigertz 1968)

Experimental and computational methods

used for constant load exercise by a variable resistor, the slider of which was mechanically linked to a cam and follower device (for details, see Wigertz 1970) The contour of the cam as described in polar coordinates was

$$r(\psi) = r_{min} + (r_{max} - r_{min}) \frac{1 - 2\psi/\pi}{\alpha + 1 + (\alpha - 1)\ 2\psi/\pi} \qquad -\pi/2 \leq \psi \leq \pi/2$$

where r is the radius of the cam, ψ is the angle of displacement, r_{min} and r_{max} determine the maximal and minimal work load (1050 and 250 kpm/min) and α is the ratio of the maximal to minimal work load In this way precise ramp function work load patterns with a preset amplitude (A=800 kpm/min) and different durations (T) could be accomplished

Recordings and experimental procedure The following variables and quantities as well as work load inputs were monitored continuously and simultaneously on an 8-channel ink recorder

on the principles of measuring the inverted time interval between successive R-waves with its value held over the next heart period *Rectal temperature* was measured by means of a thermistor probe (Yellow Springs Instruments, Inc, No 401 with amplifier Model 41 TA)

To minimize changes in stroke volume all experiments were carried out with the subjects in the supine position The subjects had previously been made familiar with the subjective sensations experienced during supine leg exercise with ramp function work load They were positioned supine on the bed with their shoulders firmly against a fixed support to avoid isometric work with the arms and with their gym shoes attached to the pedals with adhesive tape They then rested for at least 20 min before being connected to the recording instruments Except during the rest periods at the beginning and the end of the experiment the subjects pedaled

respectively

The work load profile shown in Fig 1 was the same in all experiments but was unknown to the subjects The amplitude and duration of the ramps could therefore not be anticipated The peak load and the duration of the exercise were chosen so as to be well tolerated by the subjects and to avoid any significant accumulation of lactic acid in the arterial blood As can be seen in Fig 1 the ramp changes in work load commenced with a 6 minute warmup period at 650 kpm/min and were interposed with a 5 minute exercise period at the maximal and minimal work load levels to provide stable state values at 250 and 1050 kpm/min and to ensure a

TABLE 1 Dimensional and functional data

Subject	Age (yrs)	Weight kg	Height (cm)	Blood pressure mm Hg supine	W_{150}* (kpm min) supine
HN	23	73	177	110 75	1 285
SS	23	75	180	115 70	1 851
BS	23	77	189	120 70	1 912
RS	24	81	189	135 80	1 454
JS	22	71	184	125 70	1 347
JW	25	69	173	130 80	1 236

* Work load giving a pulse rate of 150 beats min



Fig 1 Diagram of work load profile Heavy lines indicate the portions of the work load se- ... The dura- ... of 250 and ... nd negative,

reasonably stable condition before the start of a new ramp change in work load The room temperature of the laboratory was kept within 1° C and averaged 19 6° C Barometric pressure averaged 762 torr, relative humidity 50 %

Computations

The time averages of ventilation and heart rate over the 5th minute of sustained constant load

noise in the responses and the influence of the non stationary components this difference was calculated as the time average only over the last 1/4 of the ramp The analog computer analysis included logical screening and filtering (*cf* Wigertz 1970)

Results

Stable state responses Individual and mean stable-state values (5th minute) for ventilation and heart rate responses to sustained constant load exercise at 250 650 and 1050 kpm/min obtained during the early part of the experiment (depicted by heavy lines, *cf* Fig 1) are presented in Table II The mean values were 16 5, 26 3 and 41 0 l/min for ventilation and 84, 104 and 124 beats/min for heart rate

Ramp responses A segment of an original recording from subject RS is shown in Fig 2 Following the start of a positive or negative ramp change in work load, both the heart rate and the ventilation altered gradually to approach the same slope as the ramp (*cf* Fig 6) Individual and mean values of T_L are given in Table III and I

TABLE II Ventilation and heart rate during the 5th min of constant load supine leg exercise at 250, 650 and 1 050 kpm/min

Subject	Inspired minute volume (l/min)			Heart rate (beats/min)		
	250 kpm/min	650 kpm/min	1 050 kpm/min	250 kpm/min	650 kpm/min	1 050 kpm/min
HN	18 6	29 6	46 8	90	112	135
SS	18 1	25 3	39 6	80	96	112
BS	14 5	25 8	41 8	72	91	112
RS	*17 7*	*26 0*	*40 3*	*90*	*108*	*126*
JS	15 5	24 5	37 0	84	107	123
JW	*14 5*	*26 4*	*40 2*	*90*	*109*	*134*
Mean	16 5	26 3	41 0	84	104	124

for all ventilatory and heart rate ramp responses Statistical t-test analysis showed no significant differences between T_L-values for positive and negative ramp responses of either variable Therefore individual and group means have been computed using T_L-values from the corresponding positive and negative ramps For heart rate the means of these values were *84 0* sec at 114 kpm min ^{2}, *56 4* sec at *160* kpm min ^{2} and 38 8 sec at 267 kpm min ^{2}, demonstrating that the T_L-values became shorter for this variable the steeper the ramp input For ventilation (Table III) the shortening of the T_L-values was less marked the corresponding means of T_L being 77 5 sec, *63 9* sec and *57 0* sec respectively (average = *66 1* sec)

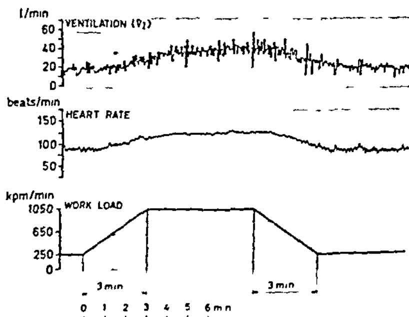


Fig 2 Segment of original record from subject RS cf Table I The recorded signals were also stored on magnetic tape for further processing and off line analysis of data Slope of positive and negative ramps corresponds to 267 kpm min ^{2}

Table III Lag time (T_L) in sec for ventilation with positive and negative ramp changes in work load between 250 and 1 050 kpm/min

Rate of work load change (slope of ramp)	114 kpm min^{-1}			160 kpm min^{-1}			267 kpm min^{-1}		
Subject	positive ramp	negative ramp	mean	positive ramp	negative ramp	mean	positive ramp	negative ramp	mean
HN	107.0	79.1	93.1	128.0	47.2	87.6	73.2	67.3	70.3
SS	69.5	81.2	75.4	28.4	42.2	35.3	29.6	46.5	38.1
BS	91.0	89.7	90.4	65.6	60.8	63.2	34.4	57.3	45.9
RS	75.3	84.9	80.1	59.4	38.3	48.9	87.0	59.3	73.6
JS	66.1	68.8	67.5	70.9	74.7	72.8	62.4	38.4	50.4
JW	60.1	57.1	58.6	95.4	55.5	75.5	67.1	60.3	63.7
mean	78.2	76.8	77.5	74.6	53.1	63.9	59.1	54.9	57.0
SD	17.6	11.9	13.2	33.9	13.4	19.1	22.7	10.5	14.3

Table IV Lag time (T_L) in sec for heart rate with positive and negative ramp changes in work load between 250 and 1 050 kpm/min

Rate of work load change (slope of ramp)	114 kpm min^{-1}			160 kpm min^{-1}			267 kpm min^{-1}		
Subject	positive ramp	negative ramp	mean	positive ramp	negative ramp	mean	positive ramp	negative ramp	mean
HN	97.6	128.4	113.0	97.4	65.0	81.2	48.2	66.0	57.1
SS	82.9	113.2	98.1	29.8	30.6	30.2	18.6	32.9	25.8
BS	48.9	101.7	75.3	34.7	58.8	46.8	35.4	36.5	36.0
RS	71.9	65.1	68.5	70.9	26.3	48.6	45.4	46.5	46.0
JS	62.3	56.8	59.6	65.1	78.8	72.0	32.2	27.1	29.7
JW	97.3	81.7	89.5	83.8	35.1	59.5	50.3	26.8	38.6
mean	76.8	91.2	84.0	63.6	49.1	56.4	38.4	39.3	38.8
SD	19.5	28.0	19.9	26.8	21.4	18.5	12.1	15.0	11.4

In order to further investigate these relations the various slopes (S) of the regression lines for the lag time (T_L) as a function of the rate of change in work load was computed for the individual data (positive and negative ramps) (Table V). For ventilation the values of the coefficient S ranged from -0.260 to $+0.003$ (Mean ± S.E. = -0.120 ± 0.041). For heart rate the S values ranged from -0.380 to -0.143 (Mean ± S.E. = -0.269 ± 0.034).

Rectal temperatures. Mean rectal temperature increased progressively during the first 50 min of the exercise session from 36.9° C to 37.7° C and then reached a stable level. The difference between observed maximal and minimal values for each subject during the experimental session ranged between 0.39 and 1.09° C, the mean difference being 0.77° C.

TABLE V. Slopes (S) of the regression of T_L as a function of rate of change in work load

Subject	*Ventilation*	*Heart rate*
HN	−0.130	−0.318
SS	−0.185	−0.380
BS	−0.260	−0.241
RS	−0.018	−0.143
JS	−0.127	−0.228
JW	0.003	−0.303
Mean	−0.120	−0.269
S.E.	0.041	0.034

Discussion

The main objectives of the present work were to explore the suitability of ramp changes in work load for studying the dynamics of the ventilation and heart rate responses to exercise and the possible influence on these responses of alterations in the slope of the work load ramp. As in previous studies of the dynamics in the responses of the above mentioned variables to exercise stimuli in the form of deterministic input functions (Wigertz 1970, Broman and Wigertz 1970), the work intensity used in the experimental protocol (see Fig. 1) had an intermittent character with the highest work levels well below the subject's maximal work capacity. It is evident from the results from stable state measurements (see Table II) that the systems underlying the ventilatory and heart rate responses may be regarded as reasonably linear. In this respect the present result are comparable to those previously obtained with sine wave and step testing.

The small rise in mean rectal temperature over the duration of the experiment combined with the fact that blood lactates did not show significant increases in a previous series of experiments (Wigertz 1970), in which the material and work load time pattern was comparable with those used in the present study, makes it unlikely that these factors were critical for the dynamics of the ventilation and heart rate changes in the present experiment. Moreover, the heart rate showed only a small drift over the duration of experiment, only one beat/min for each 9 min period as measured during stable state of the first and last periods of the highest work load of 1050 kpm/min.

In the present study T_l was determined by averaging technique over the last quarter of the work load for all slopes to obtain reasonable equivalence in the filtering of random fluctuations in the output signals. Thus, with the shortest ramps (duration = 3 min) the computation of T_l started already 135 sec following their onset. Strictly, the measurement of T_l, which is a stable state parameter, should not be performed until all transients in the response have vanished. In order to estimate the influence of remaining transients on the measured T_l values the latter were compared with those obtained from simulated dynamic ventilatory and heart rate systems having the same dynamic properties as obtained by harmonic analysis of experimental

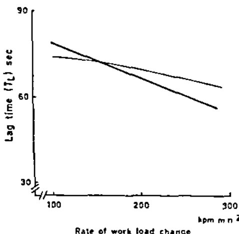


Fig 3

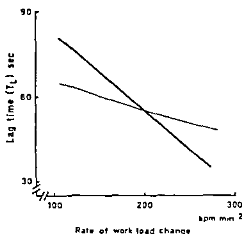


Fig 4

Fig 3 Regression line for the experimental mean ventilatory values of T_L as a function of the rate of work load change (heavy line) and predicted regression line with simulated T_L values (thin line)

Fig 4 Experimental and predicted regression lines for heart rate See further Fig 3

data as described by Wigertz (1970) These properties were obtained from 11 subjects of similar physical fitness as those of the present study, performing similar supine cycling exercise with sinusoidal work loads Fig 3 and 4 show that the negative regression was more pronounced for the present experimental data than for the simulated and that this was especially true for heart rate The slopes (coefficient S) were computed for both the experimental and simulated data From the values given in Table VI it can be calculated that the mean S values from the experimental heart rate data differed significantly ($p<0.01$) from those of the simulated data the implication being that—irrespective of any influence of remaining transients—the ex

Table VI Comparison between predicted and experimentally obtained slopes (S) of the regression line of T_L as a function of rate of change in work load

Slope ×10	Ventilation	Heart rate
From experiments		
Mean	—1 20	—2 69
S E	0 41	0 34
Predicted[1]	—0 55	—0 95
Mean difference	—0 65	—1 74
$t = \frac{\text{Mean difference}}{\text{S E}}$	—1 59	—5 12

[1] from transfer functions with sinusoidal work load inputs previously determined for athletes (Wigertz 1970)

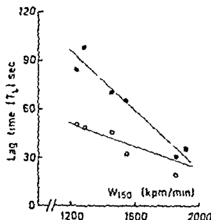


Fig 5 Individual T_L values for heart rate as a function of physical work capacity (W_{150})
Filled circles positive and negative work load ramps of 160 kpm min^{-2}
Open circles positive and negative work load ramps of 267 kpm min^{-2}
T_L for the two ramp responses were negatively correlated to work capacity *r* being −0.968 and −0.874 respectively

perimental ramp responses of the heart rate were dependent on the rate of work load change There was no corresponding dependence for the ventilatory responses the T_L values of which averaged 66 sec This agrees with the first order characteristics of the ventilatory response (*cf* below)

It has recently been shown by sine wave and step testing (Wigertz 1970 Broman and Wigertz 1970) that within the reasonably linear region of operation the systems underlying the ventilation and heart rate responses to work load are first and second order respectively When a step change in work load was used as the forcing function it was found that the time constants associated with the second order model for heart rate ranged from 9.0 to 11.7 sec and from 1.8 to 3.7 min and that the share of the component having the shorter time constant increased when the step change in work load was initiated from a lower level It may be inferred then that the shortening of T_L for the heart rate responses in the present study with increased steepness of the work load ramp suggesting a slope dependence of this variable may be due to an increase in the share of the component having the shorter time constant Thus there is an apparent similarity between the results of the step function study and those of the present ramp function study i e the faster step response associated with a low initial work level corresponds to the faster ramp response obtained with an increased slope of the work load ramp As to the nature of the mechanism underlying the slope dependence of the ramp response of the heart rate an increased influence of the centrally induced drive responsible for the fast and powerful increase of heart performance in exercise (*cf* Folkow Heymans and Neil 1965) would seem to be a very likely one Such a mechanism would strive to narrow the gap (*i e* the amplitude difference $x(t) - y(t)$ in Fig 6 between the metabolic demand of exercising muscles and the circulatory response in the ultimate control of gas exchange

Observations by Jones *et al* 1970) on the behavior of cardiac output indicate that better trained subjects reach their new stable-state values faster than more sedentary ones after step change in work load Similar observations have also been made in this laboratory (Broman and Wigertz 1970) Individual T_L-values for the

heart rate were therefore plotted against physical working capacity expressed as W_{150} (*cf* Table I) For the two highest rates of change in work load (160 and 267 kpm min^{-2}) there was a statistically significant correlation between these variables r values being respectively -0.968 and -0.824 ($r = \pm 0.811$ at the 5 % level) (Fig 5) Since in supine leg exercise the changes in heart rate parallel those in cardiac output (*cf* Wigertz 1970), this relationship supports the postulate that cardiac output responds faster the more well trained the subjects

Appendix

Consider a linear system with time invariant dynamic characteristics Let the input be time linear (a positive or negative ramp function) and applied to the system at time $t=0$ (see Fig 6)

$$x(t) = \frac{A}{T}\, t + x_0 \qquad 0 < t < T$$

where x_0 is the value of the input signal at $t=0$ and A is its increment attained at time T

Assume that the normalized transfer function of the system can be written

$$H(s) = \exp(-sT_D)\ \Sigma_i \frac{a_i}{1 + s\tau_i} \qquad \Sigma_i a_i = 1$$

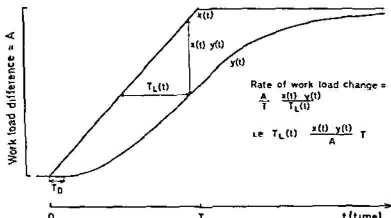
Then the output will be (Fig 6)

$$y(t) = \frac{A}{T}(t - T_D - \Sigma_i a_i \tau_i (1 - \exp(-(t - T_D)/\tau_i))) + x_0 \qquad 0 < t < T$$

where T_D is pure time delay

τ are the time constants of the system

$i = 1, 2, \ldots n$ n is the order of the system



The dynamics of
input and output
as the amplitude
With the transfer function given above $T_L(t)$ will be

$$T_L(t) = T_D + \Sigma_i a_i \tau_i (1-\exp(-(t-T_D)/\tau_i))$$

After an initial non stationary phase, the lag time is a constant parameter

$$T_L(t) \to T_L = T_D + \Sigma_i a_i \tau_i \qquad t \to \infty$$

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Changes in the Electrochemical Potential Difference for HCO_3^- between Blood and Cerebrospinal Fluid and in Cerebrospinal Fluid Lactate Concentration during Isocarbic Hypoxia[1]

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Abstract

Mines, A H and S C Sorensen *Changes in the electrochemical potential difference for HCO_3^- between blood and cerebrospinal fluid and in cerebrospinal fluid lactate concentration during isocarbic hypoxia* Acta physiol scand 1971 *81* 225—233

The changes in csf [HCO_3^-] and [lactate] were followed in dogs whose arterial pH, P_{CO_2}, and [HCO_3^-] were kept constant during 6 hrs hypoxia or normoxia. The results confirm that hypoxia causes a lowering of [HCO_3^-]$_{csf}$ We believe it is reasonable to assume that an increased anaerobic glycolysis in the brain is responsible for titrating HCO_3^- out of csf during isocarbic hypoxia although the lactate concentration in csf increased only about one third as much as the [HCO_3^-] decreased The action of hypoxia on [HCO_3^-]$_{csf}$ is expressed in terms of its effect on the electrochemical potential difference for HCO_3^- between mean capillary plasma water and csf

Changes in cerebrospinal fluid (csf) [HCO_3^-] and [H^+] are instrumental in the ventilatory adjustments to chronic blood acid base changes and to chronic hypoxia (Severinghaus *et al* 1963, Pappenheimer *et al* 1965) The mechanisms which cause the change in csf composition however still remain largely unknown

Cerebrospinal fluid is in many mammals a few mV positive relative to blood or extrameningeal extracellular fluid at a normal plasma pH (Lehmann and Meesmann 1924, Loeschcke 1956 a, Held, Fencl and Pappenheimer 1964, Kjallquist and Siesjo 1967, Sorensen and Severinghaus 1970) During normoxia with a normal

[1] A preliminary report of some of the results reported herein have been presented at the fall meeting of the American Physiological Society, Davis, California August 1969

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blood acid base status, $[HCO_3^-]$ in csf is however lower and $[H^+]$ is higher than in arterial or mean capillary plasma, indicating that these two ions are not in electro chemical equilibrium across the boundaries separating blood and csf (the blood brain barrier) This fact led to the suggestion that the concentration of these ions in csf and brain interstitial fluid was regulated by active transport mechanisms trans porting either H^+ or HCO_3^- across the blood brain barrier Pappenheimer *et al* (1965) and Fencl *et al* (1966) suggested that the activity of such an active transport system was affected by changes in $[HCO_3^-]$ or pH in plasma but recently Siesjo and Kjallquist (1969) proposed that the rate of active transport is determined by P_{CO_2} Severinghaus *et al* (1963) and Mitchell *et al* (1965) however proposed that the *transport rate of either H^+ or HCO_3^- between blood and brain extracellular fluid* was regulated by the pH in brain extracellular fluid

According to the latter scheme the changes in $[HCO_3^-]_{csf}$ during both chronic blood acid base changes and chronic hypoxia were secondary to the ventilatory changes mediated through the peripheral chemoreceptors The peripheral chemo receptors mediate acute changes in ventilation and P_{CO_2} which changes pH in brain extracellular fluid They believed that this pH change led to a change in the rate of active H^+ or HCO_3^- transport between blood and brain extracellular fluid in order to restore pH in brain extracellular fluid towards normal

Several findings could not be explained if only this hypothetical mechanism were causing the changes in $[HCO_3^-]_{csf}$ during chronic hypoxia For instance animals deprived of the peripheral chemoreceptors hyperventilate and their $[HCO_3^-]_{csf}$ is lowered during chronic hypoxia although they do not hyperventilate acutely when exposed to hypoxia (Sorensen and Mines 1970 Sorensen 1970) We therefore sug gested that an increased anaerobic metabolism in the brain is the primary event which initiates and maintains the lower $[HCO_3^-]_{csf}$ during chronic hypoxia at least in the chemoreceptor denervated animals Support for this suggestion was found in the demonstration in man of an increased brain glycolysis during acute and chronic hypoxia (Cohen *et al* 1967 Sorensen Milledge and Severinghaus 1969

The lactate (La^-) concentration in csf however increases relatively little compared to the fall in $[HCO_3^-]_{csf}$ during chronic hypoxia even in chemoreceptor denervated animals which has been advanced as an argument against the role of increased brain glycolysis in lowering $[HCO_3^-]_{csf}$ At least two factors could explain this discrepancy between the decrease in $[HCO_3^-]_{csf}$ and the increase in $[La^-]_{csf}$ during hypoxia 1 The lowering of $[HCO_3^-]_{plasma}$ due to hyperventilation and eventual renal compensa tion of the respiratory alkalosis causes a lowering of $[HCO_3^-]_{csf}$ 2 The change in $[La^-]_{csf}$ *does not reflect quantitatively the change in $[HCO_3^-]_{csf}$ from titration with H^+* formed by the brain

These experiments were performed to examine the latter possibility by measuring the changes in $[HCO_3^-]_{csf}$ and $[La^-]_{csf}$ in dogs during 6 hrs of hypoxia while arterial P_{CO_2} pH and $[HCO_3^-]_{plasma}$ were kept constant at a normal level From the results we can also calculate the effect of hypoxia on the electrochemical potential dif ferences for H^+ and HCO_3^- between blood and csf

Methods

Mongrel dogs weighing between 20 and 35 kg were used. The animals were anesthetized with pentobarbital (30 mg/kg), intubated and ventilated with atmospheric air using a Harvard pump. They were paralyzed with Flaxedil® 20 to 40 mg. Supplementary doses of pentobarbital and Flaxedil were given throughout the experiment. Airway P_{CO_2} was continuously monitored with an infrared CO_2-analyzer (Beckman LB-1) and ventilation adjusted to keep alveolar P_{CO_2} close to 40 mm Hg. Esophageal temperature was monitored with a teletermometer (Yellow Springs) and maintained constant at 38±0.2° C by appropriate use of a heating lamp and a fan. The femoral artery and vein were cannulated for sampling and infusion. The atlanto-occipital membrane was exposed and punctured with an 18—20 G Rochester needle. The metal cannula was withdrawn and the polyethylene catheter was fixed to the occipital bone. Arterial pH, P_{CO_2}, P_{O_2} and csf pH were measured using appropriate electrodes (Radiometer, Copenhagen). $[HCO_3^-]$ in arterial plasma was calculated from P_{CO_2} and pH and converted to concentrations per kg plasma H_2O assuming an 8 % solid content. CO_2 content in csf was measured a.m. van Slyke and P_{CO_2} and $[HCO_3^-]$ in csf were calculated from CO_2 content and pH (Mitchell, Herbert and Carman 1965). Blood and csf samples were precipitated in perchloric acid immediately after withdrawal and lactate and pyruvate concentrations were later determined enzymatically (Hohorst 1965).

General format of an experiment. An arterial sample was withdrawn immediately after the arterial catheter was in place and the $[HCO_3^-]_{plasma}$ was calculated from the measured pH and P_{CO_2}. Adequate amounts of 0.15 M $NaHCO_3$ were given if $[HCO_3^-]$ at this time was less than 24 meq/l. Arterial P_{CO_2} and pH were thereafter measured at least every 1/2 hr and $NaHCO_3$ was administered if necessary to keep $[HCO_3^-]_{plasma}$ between 24 and 25 meq/l.

When $[HCO_3^-]_{plasma}$ had been at this level for 1 hr, the first pair of arterial and csf samples were simultaneously withdrawn. Arterial blood was withdrawn and analyzed for pH, P_{CO_2} and P_{O_2} and a sample was precipitated in perchloric acid. About 5 ml of csf was withdrawn during a 10 to 20 min period; it was analyzed for pH and total CO_2 content and an aliquot was precipitated in perchloric acid.

During the hypoxic experiments the inspired gas mixture was then changed to obtain an arterial P_{O_2} of 25 to 30 mm Hg. During control experiments the animals were ventilated with atmospheric air throughout the experiments but other procedures were identical. Inspired gases were mixed from atmospheric air and nitrogen with flowmeters using a Pauling paramagnetic oxygen analyzer to measure the oxygen concentration. The inspired gas was mixed in an open-ended reservoir in front of the inlet gate of the Harvard pump. Arterial P_{O_2} was analyzed frequently in the beginning of the hypoxic period until the desired P_{O_2} had been [illegible]

Results

The mean values for P_{O_2}, acid base parameters and lactate concentrations in 8 hypoxic and 8 normoxic experiments are shown in Table I. The mean values for arterial P_{O_2}, pH and $[HCO_3^-]$ are calculated from all measurements within any given period. Arterial samples were taken every 1/2 hr and 3—4 samples were therefore taken within every 2 hr period in each experiment. The time course of an experiment and the constancy of the various controlled parameters is also demonstrated by the results from one hypoxic experiment (Fig. 1).

The changes in $[HCO_3^-]_{csf}$ and $[La^-]_{csf}$ during the 6 hrs of hypoxia are depicted in Fig. 2. The changes are expressed relative to the values in the pre-hypoxia period. $[La^-]_{csf}$ only increases about one third as much as $[HCO_3^-]_{csf}$ decreases. The absolute concentrations of $[HCO_3^-]_{csf}$ and $[La^-]_{csf}$ are compared during normoxia and hypoxia in Fig. 3.

When $[HCO_3^-]_{plasma}$ is maintained constant at the same level during normoxia

blood acid base status, $[HCO_3^-]$ in csf is however lower and $[H^+]$ is higher than in arterial or mean capillary plasma indicating that these two ions are not in electrochemical equilibrium across the boundaries separating blood and csf (the blood brain barrier) This fact led to the suggestion that the concentration of these ions in csf and brain interstitial fluid was regulated by active transport mechanisms transporting either H^+ or HCO_3^- across the blood brain barrier Pappenheimer *et al* (1965) and Fencl *et al* (1966) suggested that the activity of such an active transport system was affected by changes in $[HCO_3^-]$ or pH in plasma but recently Siesjo and Kjallquist (1969) proposed that the rate of active transport is determined by P_{CO_2}. Severinghaus *et al* (1963) and Mitchell *et al* (1965) however proposed that the *transport rate of either* H^+ *or* HCO_3^- *between blood and brain extracellular fluid* was regulated by the pH in brain extracellular fluid

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The lactate (La^-) concentration in csf however increases relatively little compared to the fall in $[HCO_3^-]_{csf}$ during chronic hypoxia even in chemoreceptor denervated animals which has been advanced as an argument against the role of increased brain glycolysis in lowering $[HCO_3^-]_{csf}$ At least two factors could explain this discrepancy between the decrease in $[HCO_3^-]_{csf}$ and the increase in $[La^-]_{csf}$ during hypoxia 1) The lowering of $[HCO_3^-]_{plasma}$ due to hyperventilation and eventual renal compensation of the respiratory alkalosis causes a lowering of $[HCO_3^-]_{csf}$ 2) The change in $[La^-]_{csf}$ does not reflect quantitatively the change in $[HCO_3^-]_{csf}$ from titration with H^+ formed by the brain

These experiments were performed to examine the latter possibility by measuring the changes in $[HCO_3^-]_{csf}$ and $[La^-]_{csf}$ in dogs during 6 hrs of hypoxia while arterial P_{CO_2}, pH and $[HCO_3^-]_{plasma}$ were kept constant at a normal level From the results we can also calculate the effect of hypoxia on the electrochemical potential differences for H^+ and HCO_3^- between blood and csf

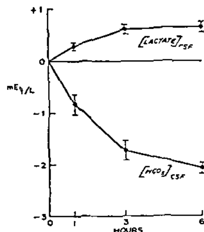


Fig 2 Changes in $[HCO_3^-]_{csf}$ and $[La^-]_{csf}$ from control values during 6 hrs hypoxia while pH and $[HCO_3^-]$ in arterial plasma was maintained constant The values are mean values from 8 expts The vertical bars indicate ± SEM

and hypoxia $[HCO_3^-]_{csf}$ increases slightly during normoxia (Fig 4) indicating that the discrepancy between the changes in $[La^-]_{csf}$ and $[HCO_3^-]_{csf}$ during hypoxia cannot be explained by too low a level of $[HCO_3^-]_{plasma}$ during the experiments

Discussion

These results substantiate that hypoxia causes a lowering of $[HCO_3^-]_{csf}$ independant of its effect on ventilation which is in agreement with the conclusion which we arrived at when studying ventilatory acclimatization to hypoxia in chemoreceptor

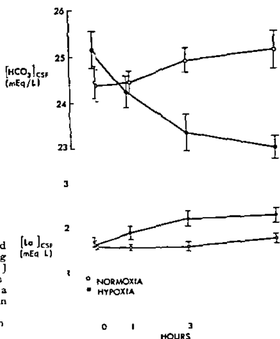


Fig 3 Changes in $[HCO_3^-]_{csf}$ and $[La^-]_{csf}$ during 6 hrs hypoxia and during 6 hrs normoxia when pH and $[HCO_3^-]$ in arterial plasma was maintained constant at the same level during hypoxia and normoxia The values are mean values from 8 hypoxic expts and normoxic expts The vertical bars indicate ± SEM

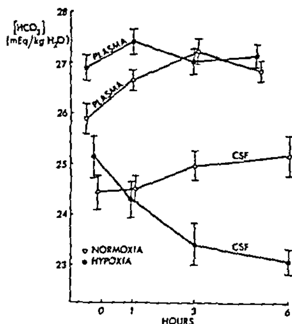


Fig 4 Arterial $[HCO_3^-]_{plasma}$ and $[HCO_3^-]_{csf}$ during 6 hrs hypoxia and during normoxia experiments $[HCO_3^-]_{plasma}$ are averaged for periods −1 to 0 hr 0 to 2 hrs 2 to 4 hrs and 4 to 6 hrs The values are mean values from 8 hypoxia expts and 7 normoxia expts The vertical bars indicate ± SEM

denervated animals (Sorensen and Mines 1970 Sorensen 1970) We suggested at that time that hypoxia lowered $[HCO_3^-]_{csf}$ by increasing the production of lactic and pyruvic acid by the brain The increased H production would lower $[HCO_3^-]_{csf}$ by titrating HCO_3^- in brain extracellular fluid This hypothesis was supported by the demonstration of an increased anaerobic glycolysis in the brain during acute and chronic hypoxia (Cohen *et al* 1967 Sorensen Milledge and Severinghaus 1969)

The decrease in $[HCO_3^-]$ in brain extracellular fluid for a certain increase in brain H production is inversely related to the permeability of the blood brain barrier to HCO_3^- and H The concomitant increase in [La] in brain extracellular fluid is a function of the permeability of the blood brain barrier to La and Lactic acid The lack of a one to-one relation between the changes in $[La^-]_{csf}$ and $[HCO_3^-]_{csf}$ during hypoxia is therefore not surprising even if the decrease in $[HCO_3^-]_{csf}$ during hypoxia was caused only by an increased brain lactic acid production

The electrochemical potential difference for HCO_3 between csf and blood During normoxia with a normal blood acid base status the $[HCO_3^-]$ is higher in the water phase of mean capillary plasma (capill) than in csf but csf is a few mV positive relative to blood indicating that HCO_3^- is not in electrochemical equilibrium across the boundaries separating blood and csf (Severinghaus *et al* 1963 Pappenheimer *et al* 1965) The electrical potential difference between csf and blood (E) increases 25 to 40 mV per unit decrease in arterial pH (Loeschcke 1956b Held *et al* 1964 Kjallquist and Siesjo 1967 Sorensen and Severinghaus 1970) but it is not affected by pH changes on the brain side of the blood brain barrier (Held *et al* 1964 Welch

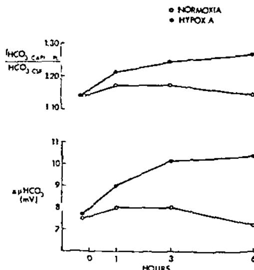


Fig 5 Changes in the electrochemical potential difference for HCO_3^- ($\Delta\mu_{HCO_3^-}$) between csf and mean capillary plasma during 6 hrs hypoxia and during normoxia with constant pH and $[HCO_3^-]_{plasma}$. For method of calculation see text and Table II

and Sadler 1965, Sorensen and Severinghaus 1970) or by hypoxia (Sorensen and Severinghaus 1970). During the present experiments E was therefore constant because arterial pH was maintained constant. The changes in concentration differences between the two compartments (Fig 4) and in the ratio of HCO_3^- concentrations (Fig 5) therefore reflect the changes in electrochemical potential differences for HCO_3^- between the two compartments. The electrochemical potential difference between csf and mean capillary plasma for HCO_3^- ($\Delta\mu_{HCO_3^-}$) may be defined

$$^{1}\Delta\mu_{HCO_3^-} = E - \frac{RT}{F} \ln \frac{[HCO_3^-]_{csf}}{[HCO_3^-]_{capill}}$$

where E is the potential difference csf—blood, R is the universal gas constant, T is the absolute temperature and F is Faradays number. The concentrations are expressed per kg H_2O.

We can calculate the electrochemical potential difference for HCO_3^- between csf and mean capillary plasma during the present experiments assuming E = 4 mV at an arterial pH of 7.40 (Held *et al* 1964, Sorensen and Severinghaus 1970) and assuming that $[HCO_3^-]$ in mean capillary plasma is 2 meq/kg H_2O higher than in arterial plasma (Table II) (see also Severinghaus *et al* (1963), Pappenheimer *et al* (1965)). The calculated $\Delta\mu_{HCO_3^-}$ during the two experimental situations is depicted in Fig 5 which demonstrates that $\Delta\mu_{HCO_3^-}$ increases during hypoxia but stays nearly constant when arterial $[HCO_3^-]$ is maintained constant at the same level during normoxia. In other words, hypoxia increases $\Delta\mu_{HCO_3^-}$.

[1] The electrochemical potential difference is most frequently defined as $\mu\Delta = zEF + RT \ln C_1/C_2$ with the dimension of work/mole (Joule/mole) (Ussing 1960, Davson 1964). The definition used here expresses the electrochemical potential difference with the dimension of Joule/Coulomb = Volt, which is equal to the difference between the measured potential (E) and the equilibrium potential for the ion ($RT/F \ln C_1/C_2$) (Katz 1966).

TABLE II Calculated electrochemical potential differences for HCO_3 ($\Delta\mu$ HCO_3^-) between csf and capillary plasma during hypoxia and normoxia with constant pH_a and $[HCO_3^-]_{a\,pl}$

		$[HCO_3^-]_{csf}$ (meq/kg H_2O)	pH_a[1]	$[HCO_3^-]_{a\,pl}$[1] (meq/kg H_2O)	$[HCO_3^-]_{cap\,m\,pl}$[2] (meq kg H_2O)	E[3] csf plasma (mV)	$\Delta\mu$ HCO_3^-[4] (mV)	$\frac{[HCO_3^-]_{cap\,m\,pl}}{[HCO_3^-]_{csf}}$
	Control	25 2	7 396	26 9	23 9	4 1	7 7	1 14
Hypoxia	1 hr	24 3	7 400	27 4	29 4	4 0	9 0	1 21
8 dogs	3 hrs	23 4	7 391	27 1	29 1	4 3	10 1	1 24
	6 hrs	23 1	7 394	27 1	29 1	4 2	10 3	1 26
	Control	24 4	7 399	25 9	27 9	4 0	7 5	1 14
Normoxia	1 hr	24 5	7 408	26 9	28 9	3 8	8 0	1 17
7 dogs	3 hrs	25 0	7 408	27 2	29 2	3 8	8 0	1 17
	6 hrs	25 2	7 411	26 8	28 8	3 7	7 2	1 14

[1] mean values for periods −1 to 0 hr 0 to 2 hrs 2 to 4 hrs and 4 to 6 hrs
[2] calculated assuming a 2 mEq kg H_2O arterial to mean capillary difference for $[HCO_3^-]_{pl}$
[3] calculated assuming E 4 mV at $pH_a = 7\,400$ and $\Delta E/\Delta pH$ −30 mV/pH unit
[4] $\Delta\mu\ HCO_3^- = E + 61 \log \frac{[HCO_3^-]_{cap\,m\,pl}}{[HCO_3^-]_{csf}}$

The maintenance of an electrochemical potential difference for HCO_3 in the normoxic animal could be explained either by an active transport system pumping HCO_3 from brain extracellular fluid to blood or H in the opposite direction or by a metabolic H source in the brain Similarly the increasing disequilibrium during hypoxia could be explained by an increased activity of an active transport system or by an increased metabolic H production

We cannot exclude that the pumping of a hypothetical active transport system is increased during hypoxia but it is difficult to imagine what could trigger an increased transport of HCO_3 from brain extracellular fluid to blood or H in the opposite direction because pH and Pco in blood was maintained constant during hypoxia and the effect of an increased active transport is to change pH and $[HCO_3]$ in brain extracellular fluid away from the normal values Therefore we believe it reasonable to assume that the increase in the electrochemical potential difference for HCO_3 between csf and blood during hypoxia with constant arterial pH and P_{CO_2} is caused by an increased metabolic H production in the brain whether this is lactic and pyruvic acid or some other organic acid

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The Effect of Iso-Carbic Metabolic Acidosis in Blood on [H^+] and [HCO_3^-] in CSF with Deductions about the Regulation of an Active Transport of H^+/HCO_3^- between Blood and CSF.

By

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Received 7 August 1970

Abstract

MINES, A H, C G MORRILL and S C SORENSEN *The effect of iso-carbic metabolic acidosis in blood on [H] and [HCO₃] in csf with deductions about the regulation of an active transport of H /HCO₃ between blood and csf* Acta physiol scand 1971 81 234—245

The changes in pH and [HCO_3] in csf of anesthetized paralyzed dogs during metabolic acidosis were compared with those changes when the blood acid base parameters were kept at a normal level Arterial and csf P_{CO_2} were kept constant at the same level during both kinds of experiments Arterial [HCO_3] was kept constant at about 25 meq/l during normal acid base experiments and at about 19 meq/l during acidosis experiments Arterial and csf samples were simultaneously withdrawn after 1 hr 3 hrs and 6 hrs The changes in csf pH and [HCO_3] were expressed as the changes in the electrochemical potential differences (csf blood) for H and HCO_3 During metabolic acidosis the electrochemical potential differences for H and HCO_3 decreased which could be explained by a decrease in the rate of an active transport of H^+/HCO_3 between blood and csf during metabolic acidosis at a constant P_{CO_2} The findings are compared with previous findings and we conclude that the rate of active transport of H^+/HCO_3 between blood
provides pH stability in t
whereas the effect is oppost
' useful during respiratory
increase in P_{CO_2} during respiratory acidosis

The cerebrospinal fluid (csf) of mammals is a few mV positive relative to blood (Lehman and Meesmann 1924, Loeschcke 1956 Held Fencl and Pappenheimer 1964, Kjallquist and Siesjo 1967 Sørensen and Severinghaus 1970) but [HCO_3] is lower and [H] is higher in csf than in mean capillary plasma indicating that these two ions are not in electrochemical equilibrium across the boundaries separating blood and csf This fact led to the suggestion that the distribution of these ions between blood and csf is determined by an active transport of either H or HCO_3 between blood and csf (Severinghaus *et al* 1963 Mitchell *et al* 1965 Pappenheimer

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et al 1965, Fencl, Miller and Pappenheimer 1966), but it may also be explained by a metabolic H^+ source in the brain

The calculated electrochemical potential differences for H and HCO_3 between blood and csf are seen to change during chronic blood acid base changes suggesting that the rate of active H^+/HCO_3 transport is affected by pH or [HCO_3] in plasma (Fencl *et al* 1966), or by pH in csf (Severinghaus *et al* 1963, Mitchell *et al* 1965) Recently it has been suggested that P_{CO_2} determines the rate of active transport during blood acid base changes (Siesjo and Kjallquist 1969)

The present experiments were undertaken to examine the effect of iso-carbic metabolic acidosis in the blood (lowering plasma [HCO_3] and pH while keeping P_{CO_2} constant) on the electrochemical potential differences for H and HCO_3 between blood and csf They do not examine the possible effects of changes in P_{CO_2} We can however test the possibility that csf pH affects the rate of active transport because, according to that hypothesis no changes in $[HCO_3]_{csf}$ should occur when Pco is kept constant during blood acid base changes (Severinghaus *et al* 1963 Mitchell 1965, Mitchell *et al* 1965)

Calculation of the electrochemical potential differences for H and HCO_3 between blood and csf requires information about both the concentration ratios and the electrical potential differences between the two compartments The latter parameter was not measured in these experiments but the electrochemical potential differences were calculated from measured electrical potential differences in the dog under comparable experimental conditions

Methods

Mongrel dogs weighing between 20 and 35 kg were used The animals were anesthetized with pentobarbital (30 mg/kg) intubated and ventilated with atmospheric air using a Harvard pump The animals were then paralyzed with Flaxedil® 20—40 mg Supplementary doses of pentobarbital and Flaxedil were given throughout the experiment Airway Pco was continuously monitored with an infrared CO analyzer (Beckman LB 1 and ventilation was adjusted to keep alveolar P_{CO_2} close to 40 mmHg Esophageal temperature was monitored with a tele thermometer (Yellow Springs) and maintained constant at 38±0 2° C by appropriate use of a heating lamp and a fan The femoral artery and vein were cannulated for sampling and infusion The atlanto-occipital membrane was exposed and punctured with an 18—20 G Rochester needle The metal cannula was withdrawn and the polyethylene catheter was fixed

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CO_2

from CO_2 content and pH using appropriate pK values (Mitchell Herbert and Carman 1965)

General format of an experiment An arterial sample was withdrawn immediately after the arterial catheter was in place and the $[HCO_3]_{plasma}$ was calculated from the measured pH and P_{CO_2} 0 15 M $NaHCO_3$ was given intravenously if $[HCO_3]_{plasma}$ at this time was less than 24 meq/l

When $[HCO_3]_{plasma}$ had been approximately 24 meq/l for one hour the first pair of arterial and csf samples were simultaneously withdrawn Arterial blood was analyzed for pH

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animals weight and infused within a 20 to 30 min period Arterial blood was thenceforth sampled at least every 1/2 hr and adequate amounts of HCl or $NaHCO_3$ were administered if necessary to keep $[HCO_3^-]_{plasma}$ at approximately 19 meq/l throughout the experiment During "normal acid base" experiments we attempted to maintain $[HCO_3^-]_{plasma}$ between 24 and 25 meq/l by infusion of $NaHCO_3$ if necessary but other procedures were identical In both groups of experiments the animals were ventilated with atmospheric air throughout the experiment

Zero time in the acidosis experiments was marked by the end of the initial HCl infusion i e 20 to 30 min after the beginning of the HCl infusion During "normal acid base" experiments zero time was marked by the end of the first csf sample

Paired arterial and csf samples were withdrawn after 1 hr 3 hrs and 6 hrs and analyzed like the control samples The sampling time refers to the middle of the sampling period

Results

The arterial and csf acid base parameters measured in 10 normal acid base and 9 acidosis experiments are shown in Table I Arterial Po_2 was measured during the experiments to assure that the animals did not become hypoxic during the prolonged anesthesia and artificial ventilation because hypoxia per se exerts an effect on csf acid base parameters (Mines and Sorensen 1970)

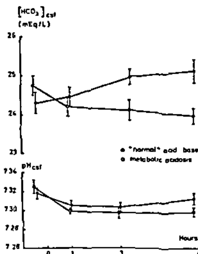
During the experiments the arterial acid base parameters were measured at least every 1/2 hr The arterial values in Table I are mean values from all measured values within any given period Therefore within a 2 hr period 3 to 4 arterial samples were obtained in any one experiment

The changes in csf pH and $[HCO_3^-]$ in the two types of experiments are also depicted in Fig 1

TABLE I Mean values SEM for arterial blood and csf parameters in the two types of experiments

		Arterial blood			Cerebrospinal fluid		
		pH[1]	$[HCO_3^-]$[1] (meq/l)	Po_2[1] (mmHg)	pH	Pco_2 (mmHg	$[HCO_3^-]$ (meq l)
Normal acid base 10 dogs	Control	7 398	23 9	84	7 318	50 5	24 3
		0 005	0 2	3	0 006	± 0 6	± 0 3
	1 hr	7 407	24 7	85	7 305	52 5	24 4
		0 003	0 2	2	±0 005	± 0 8	± 0 2
	3 hrs	7 409	25 2	83	7 303	53 9	25 0
		±0 003	± 0 2	± 1	0 005	± 0 6	± 0 2
	6 hrs	7 409	24 8	80	7 312	53 0	25 1
		0 002	0 1	± 1	0 006	± 1 0	± 0 3
Metabolic acidosis 9 dogs	Control	7 402	24 1	87	7 325	50 8	24 7
		±0 005	0 3	2	0 007	± 0 9	0 3
	1 hr	7 302	19 2	85	7 299	52 8	24 2
		±0 003	0 2	1	0 003	± 0 7	± 0 3
	3 hrs	7 298	18 7	82	7 297	52 8	24 1
		±0 004	0 2	± 1	±0 006	± 0 7	± 0 3
	6 hrs	7 296	18 7	79	7 298	52 4	24 0
		±0 004	± 0 2	± 1	±0 005	± 0 6	± 0 2

[1] Mean values for periods —1 —0 hr 0—2 hr 2—4 hr and 4—6 hrs



acidosis
during
types

Discussion

The [H⁺] and [HCO₃⁻] in csf during normoxia with a normal blood acid-base composition During normoxia with a normal blood acid-base composition csf is a few mV positive relative to blood (Lehmann and Meesmann 1924, Loeschcke 1956 Held *et al* 1964, Kjallquist and Siesjo 1967, Sorensen and Severinghaus 1970) but [H⁺] is higher and [HCO₃⁻] is lower in csf than in the water phase of mean capillary plasma indicating that these two ions are not in electrochemical equilibrium across the cell layers which separate blood and csf (Severinghaus *et al* 1963 Pappenheimer *et al* 1965, Fencl *et al* 1966) The electrochemical disequilibrium may be explained either by an active transport of H⁺ and/or HCO₃⁻ between blood and csf (Severinghaus *et al* 1963, Pappenheimer *et al* 1965) or by a metabolic H⁺ source in the brain (Mines and Sorensen 1970)

The electrochemical disequilibrium can be expressed in terms of the electrochemical potential difference for the ions which for H⁺ may be defined

$$^{1}\Delta\mu_{H^+} = E + 61 \log \frac{[H^+]_{csf}}{[H^+]_{plasma}}$$

or

$$^{1}\Delta\mu_{H^+} = E + 61 \, (pH_{plasma} - pH_{csf})$$

[1] The electrochemical potential difference is most frequently defined as $\Delta\mu = zEF + RT \ln C_1/C_2$ with the dimension of work (Joule/mole) (Ussing 1960 Davson 1964) The definition used here expresses the electrochemical potential difference with the dimension of Joule/Colomb = Volt which is equal to the difference between the measured potential (E) and the equilibrium potential for the ion (RT/F ln C1/C2) (Katz 1966)

The H and HCO_3 in csf during acidosis and alkalosis After the blood acid base composition is altered steady state concentrations of H and HCO_3 in csf are determined by 1) the changes in blood concentrations of H and HCO_3 and the accompanying change in the potential difference E (see below) and 2) by possible changes in the rate of an active transport of H and/or HCO_3 between blood and csf or by changes in the rate of H production in the brain. These latter changes would result in changes in the electrochemical potential differences for H and HCO_3 between csf and blood.

A large number of data are available from clinical and experimental studies in humans during chronic blood acid base changes. However the effect of blood acid base changes on the potential difference E in humans is not known. Therefore we cannot calculate the electrochemical potential differences from measurements of [H] and [HCO_3] in blood and csf in humans without assuming values for E but we can still examine if $\Delta\mu_H$ changes during blood acid base changes.

If $\Delta\mu_H$ remained constant in two experimental situations (′ and ″) with different blood acid base composition then

$$E' + 61(pH'_{plasma} - pH'_{csf}) = E'' + 61(pH''_{plasma} - pH''_{csf}) \qquad (1)$$

In the mammals which have been studied E varies linearly with changes in plasma pH (Held, Fencl and Pappenheimer 1966, Sorensen and Severinghaus 1970). A non linear relationship was demonstrated in the rat (Kjallquist and Siesjo 1967) but recently a linear relationship has been found also in the rat (Siesjo, personal communication). The relation between E and pH_{plasma} is described by

$$E = -F(pH_{plasma} - pH_o) \qquad (2)$$

where F is the slope of the line relating E to pH_{plasma} and pH_o is the pH_{plasma} at which the potential is zero. If we substitute for E in equation (1) using equation (2) we get

$$pH''_{csf} - pH'_{csf} = \left(1 - \frac{F}{61}\right)(pH''_{plasma} - pH'_{plasma}) \qquad (3)$$

The relationship between changes in pH_{plasma} and pH_{csf} during various blood acid base changes would therefore be described by equation (3) if the electrochemical potential difference for H stayed constant, the slope of the line being determined by the value of F.

The changes in pH_{plasma} and pH_{csf} during chronic metabolic and respiratory blood acid base changes in humans are depicted in Fig. 2. The predicted relationship if $\Delta\mu_H$ remained constant is shown in the upper left hand corner for several values of F. The results do not fall along any particular F line suggesting that $\Delta\mu_H$ does not remain constant during blood acid base changes which is in agreement with the findings in goats and rats (Fencl *et al.* 1966, Kjallquist 1970). It may be noted that the relationship between pH_{csf} and pH_{pl} is in particular different during respiratory and metabolic blood acid base changes respectively. Therefore the results could be compatible with a constant $\Delta\mu_H$ only if the F value was substantially different

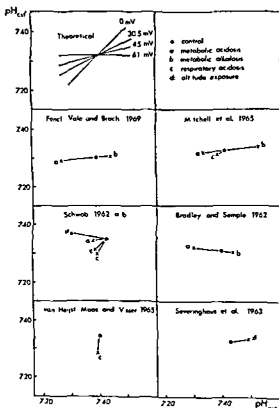


Fig 2 Changes in pH_{plasma} and pH_{csf} during clinical and experimental chronic blood acid-base changes in humans The predicted relationship between the two parameters if $\Delta\mu_{H^+}$ between blood and csf stayed constant is shown in the upper left box for several values of F (see text)

during respiratory and metabolic plasma pH changes, but in dogs only minor differences were found (Held *et al* 1964) and in rats E changes similarly during respiratory and metabolic blood acid-base changes (Kjallquist 1970) indicating a constant value for F

It has been suggested that the rate of active H /HCO_3 transport (and therefore $\Delta\mu_H$ and $\Delta\mu_{HCO_3}$) was determined by plasma pH or [HCO_3] (Pappenheimer *et al* 1965 Fencl *et al* 1966) or by csf pH (Severinghaus *et al* 1963 Mitchell *et al* 1965) or by P_{CO_2} (Siesjo and Kjallquist 1969) We may calculate $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO_3}$ between plasma and csf during the present experiments in order to examine if the results support one or the other of the above suggestions We did not measure the electrical potential difference (E) between blood and csf in these experiments but we may use values measured in dogs during acute blood acid-base changes because it has been found that E does not change in rats when plasma pH changes are sustained (Goodrich 1965 Kjällquist and Siesjo 1967) It must be emphasized that this assumption is crucial for the following conclusions

TABLE II Calculated electrochemical potential differences ($\Delta\mu$) for HCO_3^- and H^+ between csf and blood in dogs with a normal blood acid base composition and during metabolic acidosis *Arterial P_{CO_2} was kept constant at about 40 mmHg throughout the experiment in both groups of dogs*

		$[HCO_3^-]_{csf}$ (meq kg H_2O)	pH_{csf}	$[HCO_3^-]^1$ a plasma[2] (meq/kg H_2O)	pH_a^1	E (mV)		$\Delta\mu HCO_3$ (mV[3])		$^3\Delta\mu_{H^+}$ (mV)	
						a	b	a	b	a	b
Normal acid base 10 dogs	Control	24 3	7 318	25 9	7 398	4 0	4 0	5 6	5 6	8 9	8 9
	1 hr	24 4	7 30₅	26 7	7 407	3 8	3 7	6 1	6 0	10 0	10 0
	3 hrs	25 0	7 303	27 1	7 409	3 7	3 6	5 8	5 7	10 2	10 1
	6 hrs	25 1	7 312	26 8	7 409	3 7	3 6	5 5	5 4	9 6	9 5
Metabolic acidosis 9 dogs	Control	24 7	7 325	26 1	7 402	4 0	4 0	5 4	5 4	8 7	8 7
	1 hr	24 2	7 299	20 7	7 302	7 0	8 1	2 8	3 9	7 2	8 3
	3 hrs	24 1	7 297	20 2	7 298	7 1	8 2	2 4	3 5	7 1	8 2
	6 hrs	24 0	7 298	20 2	7 296	7 2	8 3	2 6	3 7	7 1	8 2

[1] Mean values for periods —1—0 0—2, 2—4 and 4—6 hrs
[2] Calculated assuming 8 % drystoff in plasma
[3] Calculated on the basis of arterial values without correcting for the difference between arterial blood and mean capillary plasma

a Calculated assuming E = 4 mV at pH_a = 7 40 and assuming that E increases 30 5 mV per pH unit fall in pH_a
b Calculated assuming E 4 mV at pH_a = 7 40 and assuming that E increases 43 mV per pH unit fall in pH_a

There is contradictory evidence about the relative effects of metabolic and respiratory plasma pH changes on E Held *et al* (1964) found in the dog a greater slope (F) during metabolic acidosis than during respiratory acidosis whereas Kjallquist and Siesjo (1967) found that E in the rat was the same during metabolic and respiratory blood acid base changes

We have therefore calculated $\Delta\mu_H$ and $\Delta\mu_{HCO_3}$ assuming both that F is similar to the value found in dogs during acute respiratory acid base changes (Held *et al* 1964 Sorensen and Severinghaus 1970) and that it is similar to the value found in dogs during acute metabolic acidosis (Held *et al* 1964) The changes in $\Delta\mu_H$ and $\Delta\mu_{HCO_3}$ during the 6 hrs of metabolic acidosis and during normal blood acid base experiments are shown in Table II and Fig 3 E was assumed to be 4 mV at an arterial pH of 7 40 and to increase 30 5 mV or 43 mV per unit fall in arterial pH

Both after 1 hr 3 hrs and 6 hrs $\Delta\mu_H$ and $\Delta\mu_{HCO_3}$ are lower in acidosis than during 'normal blood acid base conditions There is no indication of any change in the $\Delta\mu$ values after the one hour samples which suggests that a steady state is already reached 1 hr after a step change in blood composition

The $\Delta\mu$ values are calculated from assumed F values of 30 5 mV per pH unit and 43 mV per pH unit If F was larger the results could however be explained without invoking a change in $\Delta\mu$ In Fig 4 the measured changes in csf pH and $[HCO_3^-]$ are plotted as a function of the concomitant plasma changes using the 6 hr sample values The values of F which could explain the csf changes without any change in $\Delta\mu$ are shown on the respective figures The predicted relationship if $\Delta\mu$ stayed

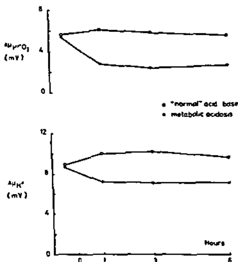
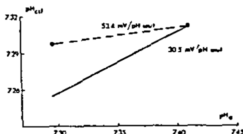


Fig 3



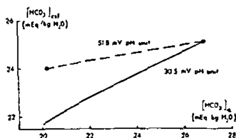


Fig 4

Fig 3 Calculated electrochemical potential difference ($\Delta\mu$) for H^+ and HCO_3 during normal acid base and acidosis experiments The $\Delta\mu$ values are calculated assuming that the potential difference csf blood increases 30 5 mV/pH unit (see Table II).

much as denoted on the respective figures

constant when F is 30 5 mV/pH unit is shown for comparison The calculated F-value, which could explain the results without any change in $\Delta\mu$, is larger than it has ever been found in dogs suggesting that $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO_3}$ changes during isocarbic metabolic acidosis

From these experiments we cannot evaluate if changes in Pco_2 affect $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO_3}$ *but the results indicate that* Pco_2 *is not the single determinant but that* changes in plasma pH or HCO_3 have an effect which is contrary to the conclusion reached by Siesjo and Kjallquist (1969)

Our conclusion agrees with the mechanism proposed by Pappenheimer *et al* (1965) and Fencl *et al* (1966) according to which the rate of active H^+/HCO_3 transport between blood and csf is affected either by plasma pH or by plasma $[HCO_3]$ changes Like those investigators we cannot distinguish between these two possibilities but in rats the correlation between $\Delta\mu_{H^+}$ and plasma pH is poor when examining respiratory and metabolic blood acid base changes (Kjallquist 1970)

In Fig 5 calculated $\Delta\mu$ values from human clinical and experimental data, and from animal experiments are plotted as a function of arterial pH and arterial

[illegible] differences for H^+ between blood [illegible] calculated as- [illegible] pH unit fall in [illegible] e author For [illegible] arterial pH except [illegible] interpretation see text

[HCO_3^-] The $\Delta\mu$ values are calculated from an assumed F value of 30 5 mV/pH unit except for the values from Kjällquist (1970), which are calculated by the author on the basis of his own potential measurements There is no correlation between the calculated $\Delta\mu_{H^+}$ values and arterial pH Whereas the correlation between $\Delta\mu_{H^+}$ and arterial $\Delta\mu_{HCO_3^-}$ is fairly good, except during altitude exposure

The inverse correlation during altitude exposure is explained by the effect of hypoxia on $\Delta\mu$ (Mines and Sorensen 1970) The data from Kjallquist (1970) were obtained after either NH_4Cl or $NaHCO_3$ injection or during respiratory acidosis from increased inspired P_{CO_2} They also combined metabolic and respiratory acidosis in various ways and from those data they concluded that changes in plasma [HCO_3^-] had no effect on $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO_3^-}$

From Fig 5 we will conclude that $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO_3^-}$ in the present experiments were affected by changes in plasma [HCO_3^-] rather than changes in plasma pH, but the results do not exclude that P_{CO_2} might also affect $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO_3^-}$ In Fig 6 the calculated $\Delta\mu_{H^+}$ values are plotted as a function of arterial P_{CO_2} demonstrating that $\Delta\mu_{H^+}$ indeed correlate equally well with P_{CO_2} It must however be emphasized that the changes in the calculated $\Delta\mu$ values are small, therefore fairly small errors in the assumptions regarding E might invalidate the conclusions

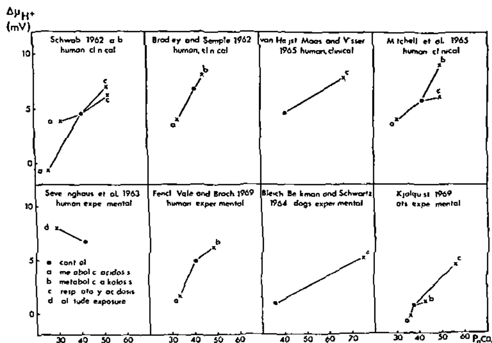


Fig 6 Changes in the calculated electrochemical potential differences for H between blood and csf ($\Delta\mu_{H^+}$) The $\Delta\mu_H$ values are calculated assuming that the electrical potential difference csf blood increases 30 5 mV per pH unit fall in arterial pH except for the data from Kjallquist (10) which are calculated by the author

The role of changes in $\Delta\mu_H$ and $\Delta\mu_{HCO_3}$ during respiratory and metabolic acid base changes in blood The changes in $\Delta\mu$ presumably reflect a change in the rate of active transport of these ions between blood and csf We might consider what the usefulness of such a mechanism might be during respiratory and metabolic blood acid base changes

During metabolic acidosis $\Delta\mu_H$ and $\Delta\mu_{HCO_3}$ decrease which diminishes the changes in csf pH and [HCO_3] for a certain change in blood pH and [HCO_3] During metabolic alkalosis the changes in $\Delta\mu_H$ are opposite in direction which also diminishes the effect of blood acid base changes on csf pH and [HCO_3] During *metabolic acid base changes the $\Delta\mu$ changes therefore diminish the changes in csf* pH and [HCO_3]

During respiratory acidosis $\Delta\mu_H$ and $\Delta\mu_{HCO_3}$ increase due to the increase in plasma [HCO_3] and/or due to the increase in P_{CO_2} During respiratory alkalosis the $\Delta\mu$ changes are opposite in direction The $\Delta\mu$ changes during respiratory acidosis and alkalosis diminish the changes in csf [HCO_3] but they magnify the changes in csf pH The changes in $\Delta\mu$ are therefore not useful in terms of pH homeostasis in the brain The usefulness of the $\Delta\mu$ changes during respiratory acidosis and alkalosis is however appreciated when considering the chain of events following an increase in P_{CO_2} due to an increase in the work of breathing

If the work of breathing is increased for instance by increasing the flow resistance to breathing P_{CO_2} increases and pH in both plasma and csf decreases If $\Delta\mu_H$ stayed constant then the relative changes in pH in csf and plasma would be determined only by the slope F of the E/pH line as depicted in Fig 2 The steady state pH values during respiratory acidosis would fall somewhere along the particular F line Renal compensation of the respiratory acidosis will however restore the plasma pH values towards normal decreasing the peripheral and the central drive to ventilation would decrease and P_{CO_2} would increase further The increase in P_{CO_2} would be limited only by the ability of the kidney to increase HCO_3 reabsorption The increase in $\Delta\mu_H$ and $\Delta\mu_{HCO_3}$ during respiratory acidosis provides a negative feed back loop which limits the increase in P_{CO_2} when the work of breathing increases

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Vascular and Lipolytic Responses in Canine Subcutaneous Adipose Tissue Following Infusion of Catecholamines

By

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Abstract

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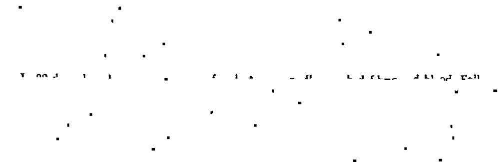
The aim of the present study was to evaluate the vascular and lipolytic effects of circulating catecholamines in canine subcutaneous adipose tissue. Adrenaline, noradrenaline or isoprenaline were added at constant rate to the arterial blood supplying subcutaneous adipose tissue *in situ*. Concentrations of added catecholamine ranged from 10^{-3} to 10^{-1} μg/ml plasma. Blood flow and the net release of glycerol were determined. Adrenaline and noradrenaline were both vasoconstrictor at concentrations above 1×10^{-2} μg/ml plasma but differed in the character of the response. Occasionally vasodilatation was observed during infusion of both noradrenaline and adrenaline. Isoprenaline caused vasodilatation at 1×10^{-2} μg/ml. Glycerol output was enhanced concomitantly with the vascular responses. The concentration of added noradrenaline (5×10^{-2} μg/ml) required to elicit a significant increase in the net release of glycerol exceeded plasma levels reported to be present in resting dogs. It appeared that blood-borne catecholamines were not as effective for lipolysis as activity in sympathetic nerves to subcutaneous adipose tissue. However, during bleeding or other extreme forms of stress the plasma catecholamine levels may be high enough to induce vasomotor as well as lipolytic responses in subcutaneous adipose tissue.

Catecholamines are potent lipolytic agents in several animal species as well as in humans (for references see Havel 1965) although their quantitative importance at physiologic plasma concentrations has not been determined. Likewise, the vascular responses of adipose tissue have not been well defined. The present experiments were performed to determine some quantitative aspects of the effects of adrenaline and noradrenaline on lipolysis and vascular resistance in the canine subcutaneous adipose tissue, when plasma levels of the added catecholamines are within physiological range. For comparison, the effect of isoprenaline was also studied.

We found that plasma concentrations of added noradrenaline and adrenaline which were effective for lipolysis were higher than the levels reported for normal resting dogs. Furthermore lipolysis was not significantly increased until vascular effects were also present.

Methods

Experiments were performed on 32 female mongrel dogs, anesthetized with sodium pentobarbital (30 mg/kg i.v.) w
subcutaneous adipose tissu
and skin as described earl
and vein were left intact
Twelve dogs were used
given i.v. after isolation of the tissue, 30—60 min before the beginning of the experimental run The tissue was perfused with blood diverted from the femoral artery via a drop chamber for measuring blood flow Venous outflow was returned to the femoral vein Drugs were in-



and end of the experiment. Glycerol was determined in arterial and venous samples The mean of the glycerol concentrations in the two samples taken from the reservoir was used as the arterial glycerol concentration

Blood flow, perfusion pressure, or systemic blood pressure, were recorded on a Grass polygraph

l Noradrenaline bitartrate, l adrenaline bitartrate or l Isoprenaline bitartrate were diluted with cold physiological saline, then infused at a constant rate (0 025 or 0 05 ml/min, for 30 min) via a sidearm in the arterial cannula. The hematocrit was determined before each infusion Plasma concentrations of added catecholamine from 10^{-3} to 10^{-2} μg/ml were attained The concentrations are expressed as the equivalent amount of free base of catecholamine

... iod In most ... was infused ... ifusions with ... ered only in constant flow perfused preparations The net release of glycerol was determined from the A V concentration difference times plasma flow The means and standard error of the means (S E) are presented The data were compared statistically by Student s t test for paired comparisons

Results

Autoperfusion

Fig 1 is the record of an experiment in which adrenaline was infused to give a plasma concentration of 0 01 μg/ml Systemic blood pressure is shown in the uppermost tracing Arterial inflow is illustrated in the middle and the data showing the net release of glycerol is plotted at the bottom of the figure In this experiment there was an increase in the net release of glycerol Initial vasoconstriction occurred in every experiment provided the plasma concentration of added adrenaline was 0 01 μg/ml, or higher, with a degree of vasoconstriction which varied according to the plasma level There was a tendency for the flow to return to or slightly exceed control levels during the course of the infusion This effect did not appear to depend upon the plasma concentration of adrenaline

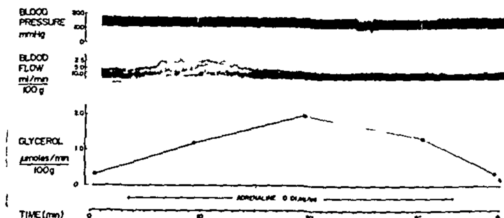


Fig 1 Data from autoperfused adipose tissue (dog) to which adrenaline was administered to give an added plasma concentration of 0 01 μg/ml The net release of glycerol is plotted at the bottom of the fig

Concentration dependent vasoconstriction was also obtained during the infusion of noradrenaline Once established it was usually maintained during the entire infusion period However occasionally a slight vasodilatation was seen during the whole infusion period This is in agreement with the results of Nielsen *et al* (1968) who found an increase in blood flow measured by the clearance of ^{133}Xe in subcutaneous adipose tissue of human subjects given noradrenaline intravenously

In most of the experiments lipolysis continued for a variable period of time after the perfusion was terminated Therefore it was decided to compute the total amount of glycerol released above resting levels In the lower part of Fig 2 the mean cumulative release of glycerol/100 g tissue is plotted Significant increases occurred when the plasma level of added noradrenaline exceeded 0 05 μg/ml The upper graph shows the per cent change of resistance during the expermnet Variability between animals was high in regard to vascular as well as metabolic responses

Constant flow perfusion

Means (±S E) from all experiments in which constant flow perfusion was employed are plotted in Fig 3 The perfusion pressure is shown in the uppermost curve Vasoconstriction was observed at concentrations of added noradrenaline and adrenaline above 0 01 μg/ml Significant changes in cumulative release of glycerol occurred at above 0 01 μg/ml of noradrenaline Maximum vascular and lipolytic responses were observed when 0 05 μg/ml was reached At this concentration noradrenaline enhanced glycerol output approximately 5 fold Adrenaline was less effective

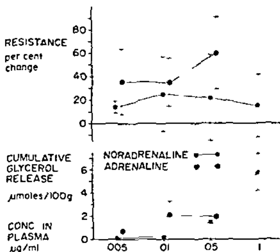


Fig 2 Means (±S E) of per cent change in resistance and of cumulative release of glycerol when adrenaline or noradrenaline was infused during autoperfusion of subcutaneous adipose tissue The responses to concentrations of added noradrenaline below 0 05 µg/ml were not significantly different from resting values (P=>05) Each point represents the mean value of 3 or 4 experiments

At plasma levels above 0 05 µg/ml the glycerol output during infusion of either catecholamine returned toward control levels Vasoconstriction, however, was maintained There was no significant difference in glycerol release between the two types of preparations when plasma levels of added adrenaline or noradrnaline were below 0 01 µg/ml

Isoprenaline

It is possible that catecholamine induced lipolysis is mediated by α- as well as β-receptor stimulation although evidence is strongly in favour of a β-type effect (Mayer, Moran and Fain 1961 Kvam, Riggilo and Lish 1965, Zsoter *et al* 1966 Fredholm and Rosell 1968) The lipolytic effects of isoprenaline are well known (Barrett 1965,

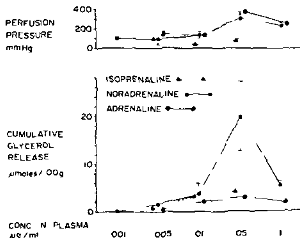


Fig 3 Means (±S E) of perfusion pressure and cumulative release of glycerol when adrenaline noradrenaline or isoprenaline were infused during constant flow perfusion of subcutaneous adipose tissue (dog) Responses to concentrations of added noradrenaline below 0 01 µg/ml were not significantly different from resting values (p=>05) Each point represents the mean value of 3 or 4 expts

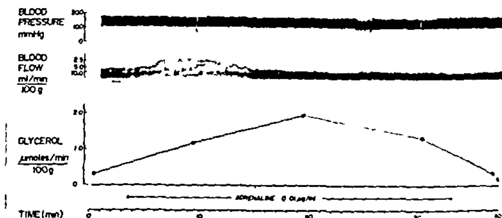


Fig 1 Data from autoperfused adipose tissue (dog) to which adrenaline was administered to give an added plasma concentration of 0 01 µg/ml The net release of glycerol is plotted at the bottom of the fig

Concentration-dependent vasoconstriction was also obtained during the infusion of noradrenaline Once established it was usually maintained during the entire infusion period However occasionally a slight vasodilatation was seen during the whole infusion period This is in agreement with the results of Nielsen *et al* (1968) who found an increase in blood flow, measured by the clearance of ^{133}Xe in subcutaneous adipose tissue of human subjects given noradrenaline intravenously

In most of the experiments lipolysis continued for a variable period of time after the perfusion was terminated Therefore it was decided to compute the total amount *of glycerol released above resting levels In the lower part of Fig 2 the mean cumulative* release of glycerol/100 g tissue is plotted Significant increases occurred when the plasma level of added noradrenaline exceeded 0 05 µg/ml The upper graph shows *the per cent change of resistance during the experiment* Variability between animals was high in regard to vascular as well as metabolic responses

Constant flow perfusion

Means (± S E) from all experiments in which constant flow perfusion was employed are plotted in Fig 3 The perfusion pressure is shown in the uppermost curve Vasoconstriction was *observed at concentrations of added noradrenaline and adrenaline* above 0 01 µg/ml Significant changes in cumulative release of glycerol occurred at above 0 01 µg/ml of noradrenaline Maximum vascular and lipolytic responses were observed when 0 05 µg/ml was reached At this concentration noradrenaline enhanced glycerol output approximately 5 fold Adrenaline was less effective

tion of circulating catecholamines to modifications of lipolysis and circulation in subcutaneous adipose tissue is significant, in dogs at least, only during stress conditions such as severe bleeding This is in agreement with recent findings by Kovach *et al* (1960) that circulating catecholamines probably play a significant role in the development of severe vasoconstriction in subcutaneous adipose tissue during bleeding

When plasma levels of noradrenaline had exceeded 0 05 μg/ml, which elicited maximum response, glycerol release began to fall (Fig 3) When the concentration was doubled lipolysis was reduced to a level that approached the response at 0 01 μg/ml This cannot be entirely explained by the extreme vasoconstriction present since a comparable increase in resistance was observed at 0 05 μg/ml One could speculate that at this concentration vasoconstriction did not reduce the flow in all exchange areas, whereas at higher plasma levels, very few of the exchange vessels were open If this were thue, only a fraction of the catecholamine would have reached the tissue to provoke increased lipolysis before vasoconstriction ensued The total resistance would be the same in either case since this in mainly regulated at the arteriolar sections, only the pattern of flow in the vascular bed would be modified That the vascular reactions may influence lipolytic activity is also suggested from the data of Scow (1965) He found that in the rat parametrial fat body, perfused with diluted blood, adrenaline in high concentrations (0 6 μg/ml) caused a strong vasoconstriction However, the release of free fatty acids was small compared with the outflow produced with corticotropin (ACTH) or thyrotropin (TSH) which were not vasoactive Moreover the reduced response at 0 1 μg/ml may be due to an auto inhibition related to the high concentration of noradrenaline This type of effect has been described in regard to FFA release from *in vitro* preparations of rat adipose tissue given noradrenaline (Wenke *et al* 1964)

It would be of interest to compare the effectiveness of circulating catecholamines with the level of control exercised by sympathetic nerve activity to adipose tissue In a previous study (Rosell 1966) in subcutaneous adipose tissue perfused at constant flow and compared in all respects to the present experiments the nerve was stimulated at frequencies considered to be within physiological range (Folkow 1952) Stimulation at 1/sec for 50 min released about 0 5 μmoles/min/100 g free fatty acids Assuming a ratio of 1 3 between glycerol and FFA and a 70 per cent re esterifica tion (Fredholm and Rosell 1970) a net release of about 0 7 μmoles glycerol/min/100 g would result A comparable increase in glycerol output occurred during infusion of noradrenaline between 0 01—0 05 μg/ml Since this noradrenaline concentration is far in excess of resting values it appears that blood borne catecholamines are not as effective for lipolysis in canine subcutaneous adipose tissue as sympathetic nerve stimulation

Weak responses to catecholamines have also been found in canine adipose tissue from a different locality Recently we have reported that noradrenaline did not increase lipolysis in mesenteric adipose tissue unless high doses (20 μg or more) were injected (Ballard and Rosell 1969) On the other hand it is possible that fat depots at other sites in the dog are more sensitive to circulating catecholamines than either

subcutaneous or mesenteric fat In this connection it is interesting to note that human omental adipose tissue has been found to be more sensitive to noradrenaline than subcutaneous adipose tissue (Carlsson and Hallberg 1968)

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The Relative Contributions of Sodium and Chloride Ions to the Conductance of Toad Skin in Relation to Shedding of the Stratum Corneum

By

ERIK HVIID LARSEN

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Abstract

HVIID LARSEN, E *The relative contributions of sodium and chloride ions to the conductance of toad skin in relation to shedding of the stratum Corneum* Acta physiol scand 1971 *81* 254—263

By means of short circuit and tracer technique, sodium transport chloride transport and D C conductance have been studied in toad skin in relation to moulting It is shown that the skin loses its permselectivity, and that active sodium c [illegible] n takes place Subsequently the passive sodium permeabili [illegible] ability The results are compatible with the hyp [illegible] the amphibian skin suppresses loss of ions along [illegible] junctions confined to the surface cells of the epithelium It is confirmed that a rate limitation of the active sodium transport is located in the outermost part of the skin

In amphibian skin, sodium and chloride are transported across the epithelial layer to the serosal side Jørgensen (1949) has shown that this transport mechanism stops functioning during moulting The net movement of NaCl is now outward despite an increased sodium influx A previous paper (Hviid Larsen 1970) deals with this phenomenon studied in the isolated toad skin during aldosterone induced shedding of the *stratum corneum* It was shown that the passive sodium permeability increased as slough formation took place, and subsequently was reestablished to the previous level The total skin conductance likewise increased, this parameter however, remained high throughout the observation period This observation can be explained by assuming a high passive chloride conductance or a high active sodium conductance towards the end of the experiment A high active sodium conductance is compatible with the fact that the rate of active sodium transport increased and remained high during the last hours of observation

In the present work, chloride conductance, sodium conductance, and total skin conductance have been studied in order to test the two possibilities just mentioned and to investigate the permselective function of the toad skin during moulting

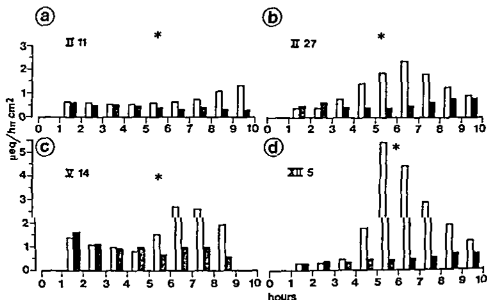
Material and Methods

The experiments were performed on automatically short circuited membranes as described previously (Hvid Larsen 1970) including the determination of tracer fluxes and the induction of slough formation When both the sodium and the chloride outflux were measured on the same skin preparation, ^{24}Na and ^{36}Cl were added to the solution bathing the serosal side of the skin and samples were taken every hour ^{24}Na was counted at once in a Packard Auto-Gamma System and ^{36}Cl was counted 3 weeks later in a Tri Carb Liquid Scintillation Counter using a toluene-ethanol scintillator The D C. resistance was calculated as the ratio of the spontaneous potential to the short-circuit current Immediately after the samples were taken the short-circuit current was interrupted and the potential recorded as soon as a stable value was reached The procedure lasted at most 2—3 sec Thus, the cellular concentrations of ions

skin the *stratum corneum* remained intact within the period of observation

During springtime and early summer reproducible results could not be obtained In the first experiment shown (Fig 1a) chloride outflux increased after shedding The chloride out

observed in double chamber experiments



Aldosterone

The experiment carried out in May (Fig 1c) is remarkable because of the high chloride outflux from the very start a phenomenon frequently observed in the period from May to July Consistent changes in chloride outflux in relation to slough formation were not seen during these months

The last example (Fig 1d) is typical of the autumn and winter experiments During the period from September to January only the diphasic response of chloride outflux in relation to moulting was found In these experiments, the shortcircuit current (SCC) increased after shedding to a level above that reached in the control membrane At other times of the year a slight increase in SCC was frequently obtained

In the experiments depicted in Fig 1a—d the toads were kept in shallow tap water at 4° C Another series of experiments has been performed in May and June with toads kept in most moss and grass on open land A steep increase in SCC after shedding (preceeded by a drop in [illegible] land

The results described in the following sections refer to experiments performed between September and December on toads kept in shallow tap water at 4° C till the day of operation

Results

1 *Slough Formation and Chloride Permeability*

The increased chloride outflux during slough formation as seen in Fig 1 can be due either to an increased chloride permeability, or, alternatively, to mucus secretion (Koefoed Johnsen *et al* 1952) which is characteristic of moulting *in vivo* (Jørgensen 1949) However mucus secretion has never been observed *in vitro* Since the net sodium flux is equal to the rate of active charge transport before as well as after slough formation (Hviid Larsen 1970)[1] an electrogen active chloride outflux can be disregarded as the source of the high chloride outflux Moreover, both chloride outflux and chloride influx undergo similar changes during slough formation as seen in Fig 2 which represents the results obtained in two pieces of skin from the same animal The observed changes in chloride outflux thus must be ascribed to change in passive chloride permeability

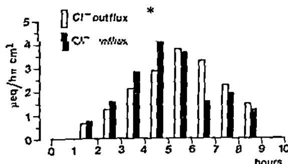


Fig 2 Chloride influx and chloride outflux in paired pieces of skin from a hypophysectomized toad Aldosterone (0 1 μg/ml) added to both preparations at zero time and slough formation completed within the hour indicated by an asterisk Σ Cl_{out} = 18 01 μeq Σ Cl_{in} = 17 87 μeq

[1] Sodium fluxes were measured in skin from toads kept in shallow tap water at 4° C during autumn and winter (group B and C)

2 Slough Formation and the Ratio P_{Cl}/P_{Na}

In order to obtain detailed information on the interdependence of Na and Cl permeabilities during moulting, these parameters were measured simultaneously on the same preparations Fig 3 illustrates the result of a single experiment 1 sodium as well as chloride outflux increase before shedding (indicated by an asterisk) 2 sodium outflux reaches a maximum 1—2 hrs earlier than does chloride outflux 3 the decrease in sodium outflux starts 2—3 hrs prior to that of the chloride outflux

The results of 8 expts are summarized in Table I, column 2 and 3 showing the mean sodium and chloride outfluxes at the second third, and ninth hour after aldosterone treatment, as well as the maximum for sodium and chloride outfluxes In addition the ratio P_{Cl}/P_{Na} was calculated for each experiment and in column 4 the mean at the respective hours of observation is given It appears that the chloride outflux always exceeds the sodium outflux and that shedding results in an increase in the ratio P_{Cl}/P_{Na} Typically, this increase in P_{Cl}/P_{Na} is preceded by a decrease to a minimum of 1.4 ± 0.1 *i e* a value close to that of the ratio between the mobilities of these ions in water (1.5 at 25° C)

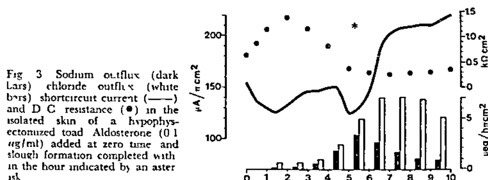


Fig 3 Sodium outflux (dark bars) chloride outflux (white bars) shortcircuit current (——) and D C resistance (●) in the isolated skin of a hypophysectomized toad Aldosterone (0.1 µg/ml) added at zero time and slough formation completed within the hour indicated by an asterisk

TABLE I Changes in the ratio P_{Cl}/P_{Na} during and after slough formation in the isolated toad skin See text for further explanation

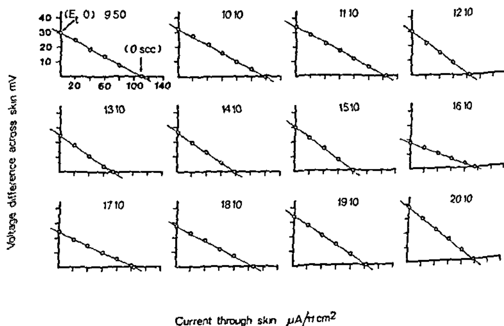
	Na outflux	Cl outflux	$\frac{P_{Cl}}{P_{Na}}$	n
	µeq/h 3.14 cm²			
2nd hour[1]	0.22 ± 0.04[2]	0.53 ± 0.05	2.7 ± 0.3	8
3rd hour[1]	0.30 ± 0.04	0.57 ± 0.04	2.1 ± 0.1	8
max Na⁺ outflux	3.34 ± 0.37	4.87 ± 0.70	1.4 ± 0.1	8
max Cl⁻ outflux	2.52 ± 0.34	5.53 ± 0.81	2.3 ± 0.4	8
9th hour[1]	1.07 ± 0.12	3.52 ± 0.65	3.7 ± 0.7	8

[1] Indicates the hour of observation after aldosterone treatment
[2] Mean ± S E M

3 Changes in D C Resistance

It has been shown earlier (Hvid Larsen 1970) that shedding is preceeded by a drop in resistance As mentioned above, the experiments were made on belly skins from toads kept in moist vegetation on open land Fig 3 reveals, that also in the present material which includes belly skins from toads kept in tap water at 4° C, the decrease in resistance is a typical response However, the resistance is evaluated on the assumption that during the entire course of slough formation, the skin may be regarded as an ohmic resistance, *i e* the transepithelial potential difference is linearly related to the current through the skin Fig 4 shows this assumption is correct The experiment was performed as follows Once every hour, a current of varying strength was applied to the preparation and the potential difference recorded For every hour the line through the points [E_t O] (open circuit potential of the skin) and [O SCC] is drawn As the values registered in between fit these lines nicely, it is concluded that the ratio E_t/SCC represents the D C resistance of the skin at the respective time of observation

In the previous paper it was shown that the resistance drop ΔR is linearly related to the resistance before shedding It appears from Fig 5 that this is also true in the present material Lines of regression from the 2 series of experiments have been drawn in the diagram The length of the full line indicates the width of variation within each series The resistance of belly skins from toads kept in moist vegetation



... the skin of a hypophysectomized ... added at 10 a m (15 30 and 16 30)

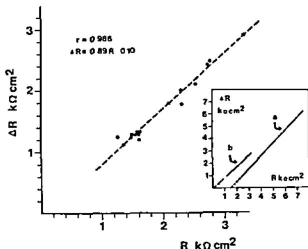


Fig 5 Resistance drop ΔR in relation to slough formation as a function of total skin resistance R prior to slough formation Toads kept in shallow tap water at 4° C.
Correlation coefficient and equation for regression line at top
$S D_{slope} = 0 04$
$S D_{intercept} = 0 08$ k ohm cm²

Inserted diagram
Line a regression line from experiments performed on skins of toads kept in moist vegetation (a r – 0 970 $\Delta R = 1 04 (\pm 0 52)R - 1 55 (\pm 0 09)$ k ohm cm² ($\pm$S.D)) Line b regression line from the present material

reaches much higher values than does that obtained in toads kept in tap water at 4° C and the resistance of the membrane after shedding is much lower in the latter group

4 The Contributions of Cl and Na to Changes in D C Conductance

The decrease in D C resistance always begins before slough formation is concluded (Fig 3 and Hvid Larsen 1970) and the same applies to the increase in sodium permeability (Fig 3) as well as chloride permeability (Fig 1d 2 and 3) Before the end of the experiments the D C resistance recovers slightly During this period sodium and chloride permeabilities decrease With respect to these parameters the entire moulting response can thus be separated into a phase of increasing conductance starting during slough formation and a phase of decreasing conductance after slough formation is concluded As the conductance (g) is determined mainly by the predominant ions i e Cl and Na it must be expected that changes in g_t are accounted for by changes in g_{Cl} and g_{Na} Sodium and Chloride being in equilibrium (for any ion the electrochemical activities are the same in the 2 solutions bathing the skin provided the skin is short circuited) g_{Cl} and the passive component of g_{Na} can be calculated using the formula (Tosteson 1959)

$$g_j = \frac{F^2 M}{RT} j —$$

Where g_j is the conductance for the ion species j M_j is the passive flux of j and F, R, and T have their usual meanings

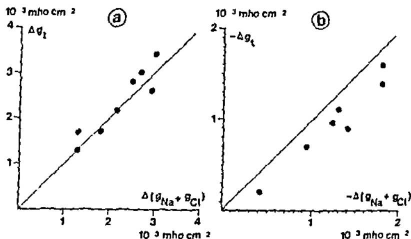


Fig 6a The *increase* in D C conductance and the *increase* in sodium and chloride conductance during slough formation

Fig 6b The *decrease* in D C conductance and the *decrease* in sodium and chloride conductance after slough formation has finished

In this way g_{Cl} and g_{Na} have been calculated for the hours of minimum and of maximum g_t and for the last hour of observation The increase in g_{Cl} and g_{Na} during the first phase as well as the decrease of these parameters during the second phase are then calculated and in Fig 6 Δg_t is depicted as a function of $\Delta(g_{Na}+g_{Cl})$ for each of the phases [1]

Fig 6a reveals that during the first phase (increasing conductance) the change in g_t is completely accounted for by the changes in the measured g_{Cl} and g_{Na} However this is not the case during the phase of decreasing conductance (Fig 6b) since all points fall below the line determined by $\Delta g_t = \Delta(g_{Na}+g_{Cl})$ Thus in the latter phase the decrease in g_{Cl} and in the passive component of g_{Na} is more pronounced than the decrease in g_t indicating that the conductance of some other skin component increases during this phase

Discussion

1 The Active Sodium Transport

As slough formation is concluded the increase in SCC observed is accounted for by an increased active sodium influx (Hviid Larsen 1969 and 1970)

Nielsen (1969) has shown that in normal *Rana temporaria* (i e non hypophysectomized animals) aldosterone treatment of skins also induced slough formation and subsequent increase in SCC (spontaneous activation period *loc cit*) During the stable period following slough formation the net sodium flux was equal to the SCC

[1] In Fig 6b one experiment has been omitted because slough formation was delayed resulting in a very short phase of decreasing conductivity within the time of observation

Characteristically, this activation period was preceded by a pronounced decrease in current (inhibition period)

In the isolated toad skin a marked decrease in current was observed (Fig 3) but in a number of experiments this decrease apparently was similar to that observed in controls (*e g* Hviid Larsen 1970 Fig 4) It therefore seems safer to state that a spontaneous inhibition of SCC is seen in the isolated skin of the toad regardless of aldosterone treatment but this inhibition is sometimes enhanced by aldosterone treatment As desquamation occurs the aldosterone treated skins become markedly different from the controls with respect to this parameter

2 *The Total Skin Conductance*

Slough formation also is reflected in total skin conductance In toad skin a phase of increasing conductance precedes the increase in SCC and is followed by a phase of slightly decreasing conductance In frog skin the increase in total skin conductance and in SCC occurred simultaneously (Nielsen 1969)

In the present study it is shown that the increase in total skin conductance as calculated from the bioelectric parameters could be ascribed to an increase in passive sodium and chloride conductance measured by mean of tracers (Fig 6a) However during the phase of decreasing total skin conductance, a conductance not identical with that of the passive sodium and chloride conductance increases (Fig 6b This is in agreement with the assumption of Hviid Larsen (1970), *ie* that the increase in active sodium influx registered after desquamation is due in part to an increased sodium permeability of the barrier separating the outside solution from the cellular compartment

Qualitatively these changes in total skin resistance are identical regardless of whether the toads are kept in terrestric or aquatic environment before isolation of the skin Both groups show a linear correlation between total skin resistance and resistance drop in connection with slough formation (Fig 5) However it is evident that the skin resistance of animals kept in moist vegetation is much higher and the resistance drop much larger as compared to that of animals kept in shallow tap water Consequently the resistance of *stratum corneum* seems to depend on the habitat in which the animals live As the resistance after desquamation is lower in skins from the aquatic group than from the terrestric group also the resistance of the deeper region of the skin may depend on environmental factors Bani (1966 observed *that skins of the toad (Bufo bufo) collected from open land have a more* compact superficial layer and a more intense keratinization than skins of the same species collected from pools However since a resistance of the isolated slough cannot be detected (Hviid Larsen unpublished) the keratinized cells proper cannot make up the structural basis of the diffusion barrier within the *stratum corneum* It is therefore more likely that the cement layer between the keratinized cells and the granulosa cells together with the occlusion belts between the keratinized cells determine the conductance of the outer region of the skin In the following section this theory is discussed

3 Slough Formation in Relation to the Functional Organization of the Skin

With respect to the observed alterations in passive sodium and chloride permeability during induced slough formation different phases can be distinguished A phase of increasing sodium and chloride permeability, one of decreasing sodium but increasing chloride permeability, and finally a phase of decreasing sodium and chloride permeability During the first phase, the skin looses its selective permeability to passively transported sodium and chloride (Table I), in this condition the preparation may be described as consisting of water filled holes permitting unrestricted diffusion of these ions From a functional point of view the skin regenerates during the following phases

In the previous paper it was proposed that the increased sodium outflux could be due to an opening of the extracellular pathway caused by the loosening of the superficial cell layer Normally this suppresses extracellular ion transport by the development of tight junction between the cells (Ussing 1969) This theory is supported by the fact that chloride outflux likewise increases and permselectivity is lost Following this line of thought it is tempting to assume that the subsequent phases are characterized by the formation of tight junctions between these cells which after desquamation are exposed to the outside solution In this manner the extracellular pathway is closed again The continued increase in chloride outflux could now be due to increased transport along the cellular pathway (this pathway is not available to passive transepithelial sodium transport) At least this is further supported by the observation that active sodium influx increases during the second phase (Fig 3) In this context it is important that Nielsen (1969) did not find any increase in sodium permeability in frog skin while an increased chloride permeability was observed in five out of six experiments this always occurred during the activation period i e simultaneously with the increased active sodium influx (thus resembling the experiments shown in Fig 1 a and c) In the terminology used above this indicates that in frog skin the extracellular pathway does not open up during induced desquamation

These considerations are an attempt at correlating the observed facta with the current view of the functional organization of the skin (Ussing and Windhager 1964 Farquhar and Palade 1966 Ussing 1969) However observations concerning structural (and chemical) details leading to desquamation are very much needed Voute *et al* (1969) by means of light microscopy followed the aldosterone induced moulting in the isolated frog skin (*Rana temporaria*) and found that prior to desquamation an increased number of cells rich in mitochondria formed small lakes between their apical part and the keratinized layer They assumed that these cells may be involved in the process which leads to the separation of the outermost keratinized cell layer In the frog skin (*Rana pipiens*) Farquhar and Palade (1965) described well developed occlusion belts both between the keratinized cells and the granulosa cells Also in the skin of *Rana temporaria* tight junctions seem to develop in the *stratum granulosum* before desquamation (Voute personal communication) The latter findings agree well with the above mentioned assumptions

As the inward transport of sodium continues during slough formation, it must be concluded that the asymmetric nature of the skin is due to processes of differentiation located beneath the cornified layer Thus the outward facing membrane ("the sodium electrode") may be formed as keratinization proceeds It is interesting to note that in the present experiments the formation of tight junctions between the outer most cells may be induced as soon as these cells are exposed to the outside solution In particular, the nature of the stimuli which trigger these membrane differentiations seems to be an attractive field of investigation

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Whole-Blood Viscosity, Hematocrit and Plasma Protein in Normal Subjects at Different Ages

By

J Ditzel and J Kampmann

Received 17 August 1970

Abstract

Ditzel J and J Kampmann *Whole-blood viscosity, hematocrit and plasma protein in normal subjects at different ages* Acta physiol scand 1971 *81* 264—268

Whole blood viscosity was measured at six fixed shear rates with a cone plate viscometer The 90 healthy subjects were evenly divided as to sex in three age groups from 15 to 80 years They were selected by the criteria normal haematocrit erythrocyte sedimentation rate serum creatinine concentration and degree of intravascular erythrocyte aggregation evaluated by conjunctival biomicroscopy and free of disease affecting known rheological properties of the blood The viscosity was higher in males than in females Age was not consistently related to whole blood viscosity except that women of the oldest age group had a higher whole blood viscosity at two isolated shear rates than women in the youngest group and the plasma fibrinogen concentration was higher in the old than in the young women

A stepwise regression analysis of rheological factors and whole blood viscosity showed that the hematocrit had by far the closest relation to the whole blood viscosity at all shear rates In the order of decreasing influence were the alpha globulins fibrinogen and serum albumin There was a sex difference in that the whole blood viscosity of men was related to the concentration of $alpha_2$ globulin whereas in women the relation was to the $alpha_1$ globulin

Few data have been published on whole blood viscosity measured with a cone plate microviscometer in carefully selected healthy subjects and the relation of age has not been investigated The purpose of our study was to measure the whole blood viscosity with a Wells Brookfield microviscometer in normal subjects of different ages and to investigate which components in blood mainly determine the viscosity

Materials and Methods

The subjects were 90 healthy persons equally divided between men and women in three age [illegible] 15 [illegible] Group I, II and III include women in the ranges 15 [illegible] and VI men in the corresponding [illegible] ital personnel and twenty patients [illegible] onents (neuroses spondyloses and fibrositis) without signs of [illegible] All subjects were selected according to the following criteria 1) a normal hemoglobin (mean 13 9 % with S D ±1 17 g% total variation for men 12 8 to 17 4 g% and for

women 11.6 to 15.0 g%), 2) a normal hematocrit (44.6 % with S.D. 2.88 %, total variation for men 42 % to 52 % and for women 37 % to 46 %), 3) a normal erythrocyte sedimentation rate (total variation 1 to 12 mm/h), 4) no or minimal intravascular erythrocyte aggregation (grade 0 to 1) observed biomicroscopically in the conjunctival vessels (Ditzel and Moinat 1959), and 5) normal serum creatinine concentration (0.9 mg%±0.14 mg%, total variation 0.6 to 1.2 mg%). The viscosity measurements were made within six hours at 37° C on whole blood stabilized with 0.1 % Ethylenediamine tetra acetic acid (EDTA). The viscosity was measured at different fixed shear rates by a Wells Brookfield cone-plate microviscometer (model LVT).[1] The shear stress was determined in duplicate at shear rates 5.75, 11.5, 23, 46, 115 and 230 sec⁻¹ (Wells, Denton and Merrill 1961). The viscometer was cali

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Results

The shear stress measurements in women and men at the six fixed shear rates show that age is not significantly related to the whole blood viscosity (Table I). Only at two rates of shear, 46 sec⁻¹ and 11.5 sec⁻¹, were there differences between group I (women between 15 and 35 years) and group III (women between 56 and 80 years)

TABLE I. Shear stress (dyn/cm^2) at six different shear rates in healthy females (group I—III) and in healthy males (group IV—VI).

	Shear stress at shear rate (sec^{-1})					
	230	115	46	23	11.5	5.75
Females						
Group I	9.7±0.99	5.3±0.52	2.5±0.24	1.5±0.16	0.9±0.11	0.6±0.09
Group II	9.4±0.98	5.3±0.57	2.5±0.31	1.5±0.25	1.0±0.17	0.6±0.16
Group III	10.0±0.87	5.5±0.50	2.7±0.27	1.6±0.20	1.0±0.14	0.6±0.11
Males						
Group IV	10.5±0.90	5.8±0.54	2.8±0.27	1.7±0.18	1.1±0.12	0.7±0.11
Group V	10.2±0.96	5.7±0.61	2.8±0.31	1.7±0.21	1.1±0.16	0.7±0.13
Group VI	10.5±1.28	5.9±0.75	2.9±0.40	1.8±0.29	1.1±0.22	0.7±0.17

TABLE II. Normal values for whole blood viscosity at six different shear rates (centipoise)

	Viscosity at shear rate (sec^{-1})					
	230	115	46	23	11.5	5.75
Females (groups I—III)	4.22±0.41	4.64±0.47	5.63±0.61	6.78±0.87	8.37±1.22	10.61±2.09
Males (groups IV—VI)	4.53±0.46	5.02±0.55	6.11±0.72	7.52±1.00	9.31±1.48	11.83±2.43
Significance	$p < 0.005$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.005$	$p < 0.02$

[1] Obtained from Brookfield Engineering Laboratories Inc., 240 Cushing Street, Stoughton, Mass., U.S.A.

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Received 17 August 1970

Abstract

Ditzel, J. and J. Kampmann. *Whole-blood viscosity, hematocrit and plasma protein in normal subjects at different ages.* Acta physiol scand. 1971. *81.* 264—268.

Whole-blood viscosity was measured at six fixed shear rates with a cone-plate viscometer. The 90 healthy subjects were evenly divided as to sex in three age groups from 15 to 80 years. They were selected by the criteria: normal haematocrit, erythrocyte sedimentation rate, serum creatinine concentration, and degree of intravascular erythrocyte aggregation evaluated by conjunctival biomicroscopy, and free of disease affecting known rheological properties of the blood. The viscosity was higher in males than in females. Age was not consistently related to whole-blood viscosity except that women of the oldest age group had a higher whole-blood viscosity at two isolated shear rates than women in the youngest group and the plasma fibrinogen concentration was higher in the old than in the young women.

A stepwise regression analysis of rheological factors and whole-blood viscosity showed that the hematocrit had by far the closest relation to the whole-blood viscosity at all shear rates. In the order of decreasing influence were the alpha globulins, fibrinogen, and serum albumin. There was a sex difference in that the whole-blood viscosity of men was related to the concentration of alpha₂-globulin, whereas in women the relation was to the alpha₁ globulin.

Few data have been published on whole-blood viscosity measured with a cone-plate microviscometer in carefully selected healthy subjects, and the relation of age has not been investigated. The purpose of our study was to measure the whole-blood viscosity with a Wells Brookfield microviscometer in normal subjects of different ages and to investigate which components in blood mainly determine the viscosity.

Materials and Methods

The subjects were 90 healthy persons, equally divided between men and women in three age [illegible] of 15 [illegible] Groups I, II and III include women in the ranges 15 [illegible] IV, V and VI men in the correspond[illegible] [illegible] or hospital personnel and twenty patients [illegible] [illegible] components (neuroses, spondyloses and [illegible] [illegible] e following criteria: 1) a normal hemo-[illegible] [illegible] [illegible] for men 12.8 to 17.4 g% and for

TABLE IV Relative significance of different rheological factors for the blood viscosity (F test F values given in brackets)

	Shear rate (sec⁻¹)					
	230	115	46	23	11 5	5 75
Males	Hematoc (61 62)	Hematoc (48 98)	Hematoc (47 71)	Hematoc (35 30)	Hematoc (40 72)	Hematoc (40 60)
	Alpha₂-gl (17 07)	Alpha₂-gl (12 00)	Fibrinogen (10 66)	Fibrinogen (8 97)	Fibrinogen (9 71)	Fibrinogen (9 93)
	Fibrinogen (9 51)	Fibrinogen (9 31)	Alpha₂ gl (10 44)	Alpha₂-gl (6 92)	Alpha₂ gl (4 09)	
	Albumin (7 91)	Albumin (6 63)	Albumin (4 40)			
Females	Hematoc (22 35)	Hematoc (19 07)	Hematoc (21 48)	Hematoc (26 53)	Hematoc (20 05)	Hematoc (23 32)
	Alpha gl (16 09)	Alpha₁ gl (13 22)	Alpha₁ gl (8 75)	Alpha₁ gl (9 04)	Alpha₁ gl (11 32)	Fibrinogen (5 00)
	Albumin (9 41)	Albumin (7 42)	Albumin (5 49)	Fibrinogen (4 72)	Fibrinogen (6 15)	
	Fibrinogen (6 62)	Fibrinogen (5 63)			Albumin (4 52)	

The relation between viscosity and the shear rate indicates that blood is an anomalous fluid The viscosity at low shear rates increased while it approached a constant low value at high shear rates Our data agree with other investigations in which a similar technique was used (Begg and Hearns 1966, Bollinger *et al* 1967 Rosenblatt *et al* 1965) Krieger and Dougherty (1959) showed that if the volume of suspended particles in a Newtonian liquid exceeds 30 % the liquid assumes anomalous qualities and this is the case for normal blood At low shear rates the erythrocytes form aggregations and thus augment the whole blood viscosity (Schmid Schonbein *et al* 1968 Chien *et al* 1967) With increasing shear rate a desaggregation takes place and the viscosity decreases to a low minimal value Plasma proteins

IV—VI)

Beta Globulin (g %)	Gamma Globulin (g %)	Fibrinogen (mg %)	Hematocrit (%)
0 76±0 14	1 09±0 21	301 3±42 4	42 6±2 3
0 65±0 11	1 01±0 25	328 5±39 1	40 9±2 6
0 77±0 05	1 17±0 18	369 7±46 9	42 3±2 2
0 64±0 10	0 93±0 13	311 3±44 5	44 8±1 9
0 68±0 10	1 01±0 25	378 7±44 4	45 4±2 2
0 73±0 09	1 13±0 16	346 3±51 6	45 5±2 9

contribute to the anomalous behaviour of blood (Schmid Schönbein *et al* 1968 Wells *et al* 1962) The relation of the plasma protein concentration to whole blood viscosity was assessed by Wells *et al* (1962) and Begg and Hearns (1966) Unlike Begg and Hearns we found that the viscosity of whole blood is related to the alpha globulins fibrinogen and albumin In men the whole-blood viscosity was related to the alpha₂-globulin and in women to the alpha₁ globulin The concentration of these protein fractions in the serum is the same in men and in women and no explanation can be offered for this difference

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From the Department of Clinical Chemistry, Malmö General Hospital University of Lund Sweden

Elimination of ^{125}I-Trypsin α-Macroglobulin Complexes from Blood by Reticuloendothelial Cells in Dog

By

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Abstract

OHLSSON K *Elimination of ^{125}I trypsin α-macroglobulin complexes from blood by reticuloendothelial cells in dog* Acta physiol scand 1971 81 269—272

Complexes of ^{125}I labelled bovine trypsin and the α_1 and α_2-macroglobulins of the dog were prepared and injected into 3 dogs The rate of elimination of the radioactivity with a half time of only 8 min fitted a single exponential curve About 50 % of the radioactivity in the whelps and about 60 % in the adult dog was recovered in the reticuloendothelial cells of the liver spleen and bone marrow

In a previous investigation (Ohlsson Ganrot and Laurell 1970 of the in vivo interaction of bovine trypsin and the plasma proteins in the dog injection of trypsin was followed by a rapid fall in the concentration of the α macroglobulins but not in that of α_1-antitrypsin Furthermore injection of a ^{125}I trypsin α_1 antitrypsin complex was followed by a transfer of ^{125}I trypsin to the α macroglobulins but injection of the ^{125}I-trypsin α macroglobulin complexes was not followed by any transfer of ^{125}I-trypsin to α_1 antitrypsin The elimination of the ^{125}I trypsin α macroglobulin complexes but not of the α_1 antitrypsin complex was shown to be a first order reaction It would therefore appear that the α macroglobulins in the dog play a central role in the elimination of trypsin and prompted investigation of the fate of the complexes

Material and methods

The basic methods used have been described earlier Ohlsson *et al* 1970) The ^{125}I trypsin α macroglobulin complexes were prepared from 150 ml of dog plasma The ^{125}I was measured in a scintillation detector of well type The autoradiographs of histological sections were obtained with the liquid emulsion technic Emulsion Ilford K 2 was used The autoradiograms were exposed for 9 days

Experimental animals

One male and one female mongrel whelp both aged 6 months and weighing 7 kg and 1 male mongrel aged 3 years and weighing 12 kg were used The animals were anaesthetized with Nembutal i v Veterinary Nembutal Abbot 60 mg/ml) They were intubated with a cuffed tube One polyethylene catheter was placed in a femoral vein and another in the brachial vein

Experimental procedure

Plasma volume was determined with Evans blue. The ^{125}I trypsin-α-macroglobulin complexes in saline corresponding to 50 ml of dog plasma was injected during 1 min into the femoral vein of each of the 3 dogs. Blood specimens were drawn one min after the end of the injection, then every second min for 10 min and finally every fifth min. 30 min after the end of the injection the animals were exsanguinated via an arterial cannula. The liver, spleen, lungs, intestines and kidneys were removed and weighed. Representative pieces were set aside for histologic examination. The rest of each organ was homogenized separately. The homogenate was weighed and 5 g of it was used for measurement of its radioactivity. Bone marrow samples of 1 g were taken from each femur and each humerus of the 3 dogs. Marrow samples were also obtained from the vertebrae. The amount of red bone marrow was assumed to correspond to the size of the liver in the adult dog and to be 50 % larger in the whelps (Bierman 1961).

Results

The rate of elimination of the radioactivity fitted a single exponential curve down to about 10 % of the initial activity with a half time of about 8 min (Fig. 1). The radioactivity recovered in each of the organs examined was calculated relative to that of the total amount of radioactive substance infused. About 80 % of the radioactivity was recovered in the liver, spleen and red bone marrow in the whelps and about 85 % in the adult dog (Table I). Autoradiographs of histological sections from these organs showed that the radioactivity was confined to cells belonging to the reticulo-endothelial system (Fig. 2).

Discussion

Several investigators (Schultze *et al.* 1963, Mehl, O'Connel and DeGroot 1964, Ganrot 1967, Lanschantin *et al.* 1966, Harpel 1970) have produced evidence that *α*-macroglobulin can bind trypsin and some other proteolytic enzymes but only

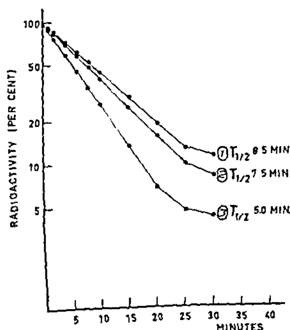


Fig. 1. Disappearance curves for 3 dogs with a preparation of ^{125}I trypsin-α-macroglobulin complexes after i.v. injection. (1) and (2) 6 months old and (3) 3 years old (logarithmic ordinate).

TABLE I. Tissue distribution of radioactivity in dogs exsanguinated 30 min after injection of ^{131}I trypsin-α macroglobulin complexes

Age of dogs	Percentage of injected dose recovered in							
	liver	spleen	bone m	lungs	intestines	kidneys	blood	carcass
6 months (2 dogs)	44.0 46.6	1.2 3.4	32.2 33.3	0.7 1.4	8.9 2.8	0.5 0.6	7.4 8.5	5.1 3.5
3 years (1 dog)	73.5	4.4	7.2	1.0	1.3	0.6	5.7	6.3

few have yielded clear evidence of such binding of an enzyme under physiologic conditions. Boyde and Pryme (1968) have described some ultracentrifugal experiments which indicate that α-macroglobulin binds cationic asparate aminotransferase, trypsin and chymotrypsin 'in conditions approaching physiological'. Observations on the binding of plasmin to α macroglobulin and the clearing of the complexes in humans (Ganrot and Nilehn 1967) seem to prove the binding of this enzyme to α-macroglobulin in vivo.

We have recently reported on studies of the interaction of trypsin and the plasma proteins in the dog in vivo (Ohlsson *et al.* 1970). It was concluded that most of the trypsin injected was eliminated from the circulation as trypsin α macroglobulin complexes. In the present investigation it was clearly demonstrated that this elimination occurs in the reticuloendothelial system. It thus seems justified to conclude that one of the functions of α macroglobulins may be the binding and clearance of proteolytic enzymes liberated in the organism.

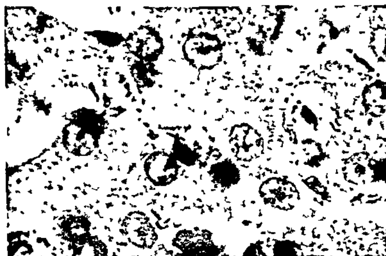


FIG. 2. Microautoradiogram of the liver of a 6 months old dog exsanguinated 30 min after injection of ^{12}I trypsin-α macroglobulin complexes. Blackening is confined practically the cytoplasm of the Kupffer cells.

It is remarkable how efficiently the body sieves can distinguish between free α-macroglobulins and trypsin-α-macroglobulin complexes which differ only little in molecular size However, the trypsin may induce conformational changes on the carrier protein The material is not large enough to warrant any conclusions about the distribution of the complexes between the individual organs of whelps and adult dogs The figures do, however, indicate that the amount of phagocytically active cells in the bone marrow and extrasplenic lymphatic tissue is larger in whelps than in adult dogs The rapid elimination of the trypsin α macroglobulin complexes limits the chances of demonstrating them even on passage of enzymes into the bloodstream The concentration of the α-macroglobulins should however presumably decrease when complexation has occurred

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On the Innervation of the Slowly Adapting Stretch Receptor of the Crayfish' Abdomen. An Electrophysiological Approach

By

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Abstract

JANSEN, J K S, A NJÅ, K ORMSTAD and L WALLOE *On the innervation of the slowly adapting stretch receptor of the crayfish' abdomen An electrophysiological approach* Acta physiol scand 1971 *81* 273—285

The synaptic effects of efferent fibres innervating the slowly adapting stretch receptor have been determined by intracellular records from reflexly intact receptors Three such efferent

The present views on signal transfer in the central nervous system are to a large extent based on the analysis of the monosynaptic reflex and certain sensory pathways in the cat To expand our field of vision it should at the present stage be useful to establish other experimentally accessible simple neuronal subsystems as examples of the *signal processing capacity of chains of neurones The abdominal stretch receptors of* the crayfish present several useful features Their peripheral location allows direct visual observation They can be adequately activated by stretch of their receptor muscles Their basic properties as receptors and neurones have been determined (Eyzaquirre and Kuffler 1955 a b) The main features of their innervation have been described anatomically (Alexandrowicz 1967) Finally their activation gives rise to well defined reflex excitation of other neurones (Eckert 1961 Fields 1966 Fields, Evoy and Kennedy 1967 Jansen Njå and Walloe 1970 a b)

The present paper gives an account of the synaptic effects of efferent nerve fibres innervating the slowly adapting stretch receptors (MRO_1) Three such fibres have been described anatomically by Alexandrowicz (1951 1967) One of these is the

well known thick accessory fibre Its synaptic effects and inhibitory action was established by Kuffler and Eyzaguirre (1955) Less direct evidence has been presented for the inhibitory nature of a second of these efferent fibres (Burgen and Kuffler 1957 Jansen *et al* 1970 a) This is confirmed by the present observations which also demonstrate the inhibitory effect and synaptic actions of the third of the efferent fibres

Some of the observations have already been presented in a preliminary form (Jansen *et al* 1970) A more detailed account of the reflex activation and effects of the thick accessory fibre is given in the subsequent papers (Jansen *et al* 1971 a, b) In the description the nomenclature of Alexandrowicz (1967) will be followed throughout

Methods

The experiments were performed on Norwegian fresh water crayfish (*Astacus fluviatilis*) which was kept at the Institute in running tap water The crayfish was decapitated and the abdomen separated from the thorax. The isolated abdomen was kept in a bath of oxygenated saline [illegible] a) The bath temperature was controlled [illegible] the temperature was kept between 8 and [illegible] eous activity of the preparation might

The stretch receptors of the 2nd 3rd and 4th abdominal segments were exposed bilaterally from the dorsal side Initially the reflex activity of the preparation was mapped by "en passage" records with suction electrodes on the dorsal nerves supplying the stretch receptors Reflexly active receptor organs were then isolated kept in a pair of forceps and moved laterally while the nerve connecting the receptor with the abdominal ganglion remained intact The removal of the receptor organ permitted transillumination which improved the view and subsequent penetration of the slowly adapting receptor In other preparations the receptor organ was left *in situ* and its observation improved by removal of the neighbouring parts of the extensor muscles In such preparations neighbouring stretch receptors were more easily accessable for stretch activation

Slowly adapting stretch receptors were penetrated by conventional glass microelectrodes filled with 1 5 M potassium citrate or 2 8 M potassium chloride Fig 1 shows the essentials of the experimental setup The microelectrode was placed in a Wheatstone bridge circuit so that the membrane potential changes of the receptor could be recorded while current was passed through the electrode The pulse generator was connected to the microelectrode through a 250 or 100 MΩ series resistor to ensure constant current conditions The current was recorded by an operational amplifier inserted between the bath and earth The signal from the micro-

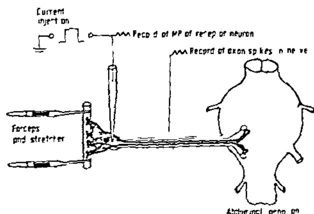


Fig 1 Diagram of experimental arrangement. The rapidly adapting receptor with its receptor muscle was usually included in the preparation but has been left out for clarity

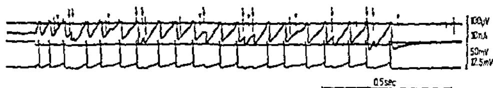


Fig 2 Reflex activation of thick and thin accessory fibre from their own stretch receptor Bottom trace DC record of membrane potential of receptor activated by current step shown above Top trace Spike activity of dorsal nerve Large accessory spike marked with arrow Small accessory spikes marked with arrow head Second trace Higher amplification AC record of receptor membrane potential Receptor spikes too large and too faint to reproduce Note large accessory IPSPs preceded by large accessory spikes in nerve record and small accessory IPSPs preceeded by small accessory spikes Two unidentified spikes in nerve ercord one large and one medium sized seen for instance near the end of the record Microelectrode filled with potassium citrate

electrode was connected through a unity gain high impedance input stage usually to a DC amplifier with a small gain and to an AC channel with 10 times higher gain The time constant of the AC channel was 1 sec The high frequency response of the entire recording system was such that the response to a square current pulse reached 90 per cent of its final value in 200 μsec The various signals recorded were displayed on a Tectronix 565 oscilloscope and usually photographed on moving film with a film speed of 10 cm sec

Results

In our hands stretch receptor inputs have been particularly useful for reflex activation of the accessory fibres Selective activation of a slowly adapting stretch receptor (MRO_1) is conveniently achieved by an intrasomatically injected depolarizing current A record from such an experiment is illustrated in Fig 2 The record of the dorsal nerve (top trace) shows that several nerve fibres in the nerve are excited by this input Of these only the ones establishing synaptic contact with the MRO_1 can be functionally identified in the present type of experiments The others are probably motor fibres for the superficial extensor muscle (Fields, 1966) which is denervated in the present set up

One of the reflexly activated spikes (marked with arrow) has a prominent effect on the membrane potential of the MRO_1 (second and fourth trace) It is followed after a latency of some five msec by a rapid hyperpolarizing deflection which interrupts the prepotential and repolarizes the receptor From the conduction velocity of this fibre its axonal spike amplitude its pattern of activation (*cf* Jansen *et al* 1970 b) and its powerful inhibitory effect it can safely be identified as the thick accessory fibre of Alexandrowicz (1967) This fibre is regularly excited by a stretch receptor input in our preparation (see Jansen *et al* 1971 b) and its identity can easily be established from its large IPSPs

In some experiments a second smaller efferent spike is also activated by a MRO_1 input This smaller spike has been marked by an arrowhead in the records of Fig 2 It is followed after a latency of some 10 msec by a small hyperpolarizing potential in the receptor The hyperpolarization and its delaying effect on the subsequent MRO_1

the smallest spike to its IPSP was just over twice that of the small accessory fibre 2) In two other preparations we have seen a comparably small and slowly conducting spike associated with the spontaneous IPSPs 3) The spontaneous IPSPs have not been activated from any ipsilateral stretch receptor input whereas the small accessory fibre regularly is 4) The spontaneous IPSPs often have a characteristic time course with a rather slow rising phase (Fig 3) The reason why we often fail to record the smallest spike from the nerve is probably that it is lost in the noise on account of its small amplitude

The spontaneous activity of this third inhibitory fibre was usually between 0 5 and 5 per sec It has varied within these limits during the period of recording from one receptor As mentioned we have not been able to activate this fibre by any ipsilateral stretch receptor input In some but not all preparations it has been moderately activated by stretch of contralateral stretch receptors in the same and neighbouring segments In addition it can regularly be activated by mechanical stimulation of the tail of the crayfish This is always a powerful stimulus in our preparations and may activate a number of different units in the dorsal nerve including the three efferent fibres to the MRO_1

Mechanisms of synaptic effects The data presented above demonstrates the existence of three efferent nerve fibres with synaptic connection with the MRO_1 One is immediately faced with the question of the possible significance of this elaborate innervation of the MRO_1 Approaching this problem we have performed experiments relevant to the questions of the mechanism of generation of these IPSPs the inhibitory efficiency of the different IPSPs and related to this the possibility of a differential location of the synapses of the three efferent fibres on the soma dendritic complex of the MRO_1

The reversal potentials of the IPSPs As seen from Fig 2 the amplitudes of the large and small accessory IPSP depend on the membrane potential of the receptor The later an IPSP occurred during the depolarizing prepotential the greater its amplitude This was confirmed in experiments with steady state changes in membrane potential produced by intrasomatically injected currents Sample records from such an experiment are shown in Fig 4 A The thick and thin accessory fibre were activated by stretch of the MRO_1 of the neighbouring segment At the resting potential both fibres produced hyperpolarizing IPSPs in the receptor That of the thick accessory fibre was about 2 5 times larger than that of the thin With a large hyperpolarizing current (7 5 nA) the two IPSPs were both reversed to depolarizing potentials An intermediate hyperpolarizing current (3 5 nA middle record) gave a membrane potential which just corresponded to the reversal potentials of both IPSPs and the two accessory spikes gave no discernible potential deflections These relationships are presented in Fig 4 B where the peak amplitudes of the two types of IPSPs are plotted against the intensity of the injected currents It appears that there is a linear relationship between IPSP amplitudes and current intensity in the hyperpolarizing direction This agrees with the linear current voltage relationships of these receptors for hyperpolarizing currents (Nakajima and Onodera 1969 confirmed in

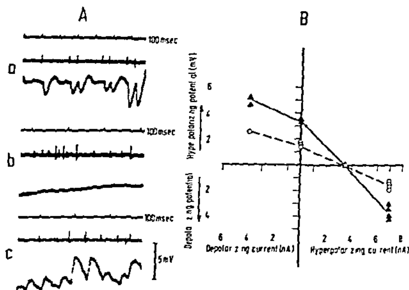


Fig 4 Reversal potentials of large and small accessory IPSPs A Records of nerve potentials (above) and AC record of receptor membrane potential (below) during stretch activation of neighbouring ipsilateral slowly adapting receptor at different levels of receptor membrane potential a No current b 3 5 nA hyperpolarizing current c 7 nA hyperpolarizing current B Peak amplitudes of large accessory IPSP (▲) and small accessory IPSP (○) (ordinate) plotted against the intensity of transmembrane current (abscissa) The different values at each level of membrane potential indicate the range of variation in IPSP amplitudes Microelectrode filled with potassium citrate

the present experiments) In the depolarizing direction the increase in IPSP amplitudes was less than linear This is probably explained by a decrease in membrane resistance (delayed rectification Nakajima and Onodera 1969)

The experiment presented in Fig 4 is in agreement with data previously published on the large accessory IPSP indicating that this is a synaptic inhibition mediated by a chemical transmitter and having a well defined reversal potential (Kuffler and Eyzaguirre 1955 Hagiwara *et al* 1960) It further shows that the small accessory IPSP also is chemically mediated and has the same reversal potential This suggests that the two IPSPs are generated by the same transmitter and that the two groups of synapses are located at equal electrotonic distances from the intrasomatically placed electrode

Fig 5 gives the same type of data for the large accessory and the spontaneously occurring IPSPs recorded in another preparation Again it is seen that there is a linear relationship between IPSP amplitudes and the membrane potential of the receptor Extrapolating the two curves show that they have the same intercept with the abscissa, *i e* the reversal potentials of the two types of IPSPs are equal Accordingly one reaches the same conclusions for the spontaneously occurring IPSPs as those stated above for the small accessory IPSPs

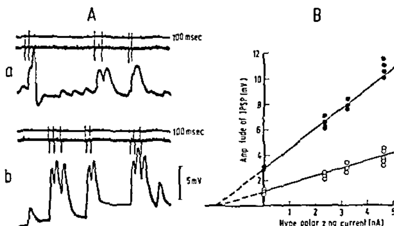


Fig 5 Amplitudes of large accessory IPSP and spontaneously occurring IPSP at different levels of re... memb...

A ... ng receptor
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Chloride ion injections Hagiwara *et al* (1960) have shown that the reversal potential of the large accessory IPSP is partly determined by the internal concentration of chloride ions in the receptor neurone With intrasomatic chloride injections one might expect a chloride concentration gradient along the dendritic processes which might produce a differential effect on differently located synapses (*cf* Burke Fedina and Lundberg 1968)

Recording electrodes filled with potassium chloride usually caused a reversal of both the large accessory IPSP and the spontaneously occurring IPSP from hyperpolarizing to depolarizing potentials within some minutes after cell penetration on account of the chloride diffusing out of the electrode An example is shown in Fig 6 Initially the large acccessory IPSPs were small and hyperpolarizing the receptor The spontaneously occurring IPSPs were just visible as small hyperpolarizing potentials *About 10 min later both types of IPSPs were depolarizing and of greater amplitude*

The amplitudes of the depolarizing IPSPs might continue to grow for about 20 min and they increased in amplitudes after extra chloride had been injected into the receptor by prolonged hyperpolarizing current pulses Quite often the large accessory IPSPs would produce a depolarization sufficient to fire the receptor (Jansen *et al* 1971 a) The reversed IPSPs recorded with chloride electrodes were dependent on the membrane potential in a way similar to those recorded with citrate electrodes (Fig 5) In the diagram of Fig 7 the amplitudes of the spontaneously occurring

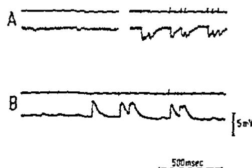


Fig 6 Reversal of IPSPs during recording with microelectrode filled with potassium chloride A About two minutes after penetration of slowly adapting receptor Just visible spontaneously occurring hyperpolarizing IPSPs in left hand part of the record Large accessory reflex elicited by stretch of neighbouring stretch receptor (right hand part of record) The small waves (50 c/sec) are due to bad recording conditions B About ten minutes after penetration Both types of IPSPs were now depolarizing

IPSP and the large accessory IPSP recorded at the same time have been plotted against each other The observations were done during prolonged recording from one receptor at a number of different levels of membrane potential and at different internal chloride concentrations Within the degree of accuracy the amplitudes of the two IPSPs are linearly related to each other Accordingly the ionic mechanisms of the two IPSPs appear to depend on internal chloride concentration to the same extent, and there was no evidence for a differential distribution of the two types of synaptic endings over the soma dendritic complex of the receptor

For the small accessory IPSPs we have no observations on its dependence on internal chloride concentration

Time course of the IPSPs In general the time course of a synaptic potential is determined by the time course of transmitter action the membrane and cable properties of the postsynaptic neurone and the localization of the presynaptic terminals on the cell We have so far not completed a full analysis of these factors but a few relevant observations deserve mentioning in spite of the difficulties in obtaining reliable measures of the time course of the two smaller varieties of IPSPs in the stretch receptor

The large accessory IPSP usually had rise times between 6 and 10 msec The time course of its falling phase was usually similar to the time course of decay of a hyperpolarizing transmembrane current pulse A typical time constant of decay might be about 20 msec and the time course was well described by a single exponential over

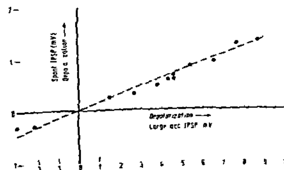


Fig 7 Covariation of amplitudes of spontaneously occurring IPSP and large accessory IPSP All data from the same receptor at different levels of internal chloride concentration at different levels of membrane potential Simultaneously observed peak amplitudes of spontaneously occurring IPSP (ordinate) and large accessory IPSP (abscissa) have been plotted against each other

the greater part of its extent In other receptors the falling phase of the IPSP was definitely slower than that of a hyperpolarizing pulse transient

The time course of the small accessory IPSP was more difficult to measure, but with the present degree of accuracy its rise time as well as its duration at half of the peak amplitude was similar to that of the large accessory IPSP The spontaneously occurring IPSP, on the other hand, differed from the other two in a characteristic way Its rise time was definitely greater This varied from receptor to receptor and in different cells we have found that the rise time of the spontaneous IPSP has been from 50 per cent to 150 per cent longer than that of the large accessory IPSP of the same receptor This was associated with a less pronounced prolongation of the time course of decay of this IPSP Its duration at half amplitude might be 20 to 50 per cent longer than that of the large accessory IPSP

Discussion

Identification of the fibres The present paper demonstrates the inhibitory function of the three efferent nerve fibres with synaptic effects on the MRO, In methylen blue preparations three such fibres have been described by Alexandrowicz (1951, 1952, 1967) Anatomically the fibres are distinguished by their diameter and partly by their peripheral terminations Two of the fibres were called the thick and thin accessory nerve according to their fibre size The third and thinnest fibre innervates the superficial extensor muscle in addition to its innervation of the receptor neurons, *and it has been designated fibre x' by Alexandrowicz (1967)*

Of the three efferent nerve fibres identified functionally in the present work the most rapidly conducting one is undoubtedly the thick accessory fibre of Alexandrowich Its synaptic effects agrees with those already described for this fibre by Kuffler and Evzaquirre (1955) For the identification of the other two we rely entirely on the difference in spike to IPSP latency of the two fibres This latency difference is presumably mainly due to differences in conduction velocity between the two fibres and therefore to differences in axonal diameter This is supported by the differences in axonal spike amplitudes Accordingly we suggest that the small fibre reflexly activated from ipsilateral stretch receptors (Fig 2 3) is the thin accessory fibre and that the spontaneously occurring IPSPs are generated by fibre x A more direct and satisfying indentification of fibre x would be a demonstration of synchronous synaptic potentials in the receptor and in superficial extensor muscle fibres

Mode of action The membrane potential dependency of the three types of IPSP provides good evidence that they are all caused by an increase in conductance of the postsynaptic membrane presumably due to the action of a transmitter substance The similarity *in reversal potentials* (Fig 4 5) and the dependence on internal chloride concentration (for the large accessory and the spontaneous IPSP) suggest identical ionic mechanisms for the three IPSPs and presumably the same transmitter agent For the large accessory IPSP there is already evidence that this transmitter GABA (Kuffler and Edwards 1958) as for the inhibition of muscle fibres in Crustacea (Otsuka, Kravitz and Potter 1969)

Distribution of synapses Some of the observations presented are relevant to the question of a possible differential location of the synapses of the three inhibitory fibres Of the three IPSPs the time course of that of the large accessory fibre was most accurately measured Its falling phase had a time course that was closely similar to or slightly slower than that of the membrane potential transient to a hyperpolarizing current step Assuming that the time course of decay of the IPSP is mainly determined by the "time constant" of the receptor membrane this my suggest that the transmitter effect is "uniformly" distributed over the soma and dendrites of the receptor Time courses of decay slower than that of the membrane potential transient might be explained by remaining transmitter action Or, alternatively, accepting the cat motoneurone as a provisional model, such IPSP shapes might be obtained from synapses located electrotonically about half way between the soma and the terminal dendrites (Rall *et al* 1967 Fig 2) The time course of the small accessory IPSPs were similar to that of the large, and this suggests they were generated in a similar manner The slower time course of the spontaneously occurring IPSPs indicates either a prolonged duration of transmitter action or a more distal dendritic location of its presynaptic terminals

The observations on the membrane potential dependency of the IPSPs showed a similar reversal potential for all three IPSPs (Fig 6 7,) This is particulary significant for the slow spontaneous IPSP and it presumably excludes an exclusive distal dendritic location of its synapses This was supported by the effect of increased internal chloride which had equal effects on the large accessory and the spontaneously occurring IPSP (Fig 8) Burke Fedina and Lundberg (1968) were able to show a differential reversal of two types of IPSPs in motoneurones on chloride injections the one presumably with somatic synapses the other with proximal dendritic synapses

Considering also the electronmicroscopical finding of synapses widely distributed over the soma dendritic complex of these receptors (Petersen and Pepe 1967) the simplest suggestion to make is that the synaptic terminals of the 3 types of inhibitory nerve fibres are intermingled over the entire synaptic surface of the receptor This is supported by the highly non linear summation of these IPSPs The two smaller IPSPs are entirely abolished when they occur near the peak of the large accessory IPSP If for instance they terminated on different dendrites one might expect a more linear summation

Some comments on the accuracy of the determination of synaptic location from the reversal potentials migh be appropriate In a steady state polarization of the soma the membrane potential decays exponentially along the dendrites if the neurone is assumed equivalent to a long cable Therefore the true reversal potential of a dendritic synapse will be smaller than the reversal potential observed in the soma by a factor e^{x}, where x is the electrotonic distance of the synapse from the soma (Calvin 1970) It appears a fair estimate that the reversal currents which are proportional to reversal potentials could be determined with an accuracy of + 15 % or better in the present experiments Accordingly the effective electrotonic distance (x'—x") between the different types of synapses was equal to or less than 0 3,

as obtained from the equation $0.85\, e^{x'} = 1.15 e^{x'}$ To transform this to anatomical distances one has to know the cable properties of the receptor For nerve cells with dendritic arborizations this has been determined adequately only for cat motoneurones (Rall 1970 Lux Schubert & Kreutzberg 1970) For the stretch receptor, the dendrites are appreciably less than 0.5 mm compared to 1 mm or more for the motoneurone The input resistance of the stretch receptor cells is usually two three times that of the motoneurones (Nakajima and Onodera 1969 personal observations) and their membrane potential transients are usually well described by a single exponential All this suggests that their 'electrotonic length may be appreciably shorter than that of motoneurones which appears to be between 1.5 and 2. Therefore one can not exclude an appreciable anatomical separation between the 3 different groups of synapses on the stretch receptor, but any such separation is unlikely *to be functionally important since they appear to be evenly distributed elec*tronically

This last point is supported by observations from one receptor for which we initially determined the inhibitory effects of the large and small accessory fibres on stretch induced firing by extracellular recording and later recorded the two IPSPs intracellularly One might suggest that even though such IPSPs were electrotonically indistinguishable, they might act differently on a generator current induced in the dendrites by stretch and on an intrasomatically injected current This however was apparently not so the ratio of the delays in MRO_1 firing caused by the two accessory fibres in stretch induced activity corresponded approximately to the ratio of amplitudes of their IPSPs

Synaptic efficiency The most striking difference between these three IPSPs is the difference in synaptic efficiency A possible measure for synaptic efficiency is the peak increase in conductance by the transmitter released by a single presynaptic impulse A relative measure of this peak conductance measured in the soma is the slopes of the curves relating IPSP amplitudes to soma membrane potential (Fig 4 5) But since the IPSPs all have the same reversal potential their peak amplitudes measured at the same level of membrane potential is directly proportional to the peak conductances In three receptors with reliable record of the large and small accessory IPSP the ratio of their amplitudes were about 7 1 5 1 and 2.5 1 respectively In 12 receptors we have simultaneous records of the large accessory IPSP and the spontaneously occurring IPSP The average ratio between their amplitudes were 5.2 1 with 2 1 and 10 1 as extreme values The mode of action of the large accessory IPSPs are discussed in greater detail in the subsequent paper (Jansen *et al* 1971 a) Assuming that the two small IPSPs act in essentially the same way as the available observations suggest it appears reasonable to accept the amplitude ratios given as a fair measure of the relative inhibitory efficiency of the three types of IPSPs

Functional significance The most challenging problem in the present investigation is the functional significance of the elaborate efferent control of the stretch receptor Unfortunately the present study gives no direct help *in this respect* Some of our

observations may, however, be relevant to this question It has often been suggested that weak synapses may have more of a biassing effect whereas more powerful synapses might have a more direct control over postsynaptic cell firing (Rall 1964) Such a biassing function might most easily be visualized for the spontaneously occurring IPSP with its small amplitude and prolonged duration of transmitter action One possibly important function for both the small IPSPs is their ability to change the firing rate of the receptor with only minor changes in its usually regular firing pattern The large accessory IPSP, on the other hand, will necessarily introduce a marked irregularity of firing To be of importance this would require pattern sensitive central neurons and these have so far not been described

Differences in function should reasonably be reflected in differences in reflex connections Our main observations in this respect are that both accessory fibres are reflexly activated from their own and neighbouring ipsilateral MRO_1 A constant observation is that the central gain of the reflex from MRO_1 to the thick accessory neurone is higher than that to the thin accessory neurone (Jansen *et al* 1971 b) We have never seen any influence on the spontaneously occurring IPSPs by ipsilateral MRO_1 impulses All three efferent fibres can be activated from contralateral MRO_1 and also by mechanical manipulation of the tail

In an earlier paper we found that small efferent fibres were more easily activated from the contralateral MRO than were the large accessory fibre (Jansen *et al* 1970 b) At that time the spontaneously occurring IPSP was not known Now it appears likely that the crossed small spike activation which then was interpreted as thin accessory fibre spikes might have consisted of thin accessory fibre spikes in some experiments and of spikes generated by the fibre x in others

The three types of efferent fibres to the stretch receptor accordingly have a partly separate and partly overlapping reflex input The existence of the thick accessory fibre is perhaps justified by its control of the receptor sensitivity (Jansen *et al* 1971 a) The two small IPSPs would in comparison have a negligible effect in this respect Different types of experiments appear to be required to shed light on their possible function

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Quantitative Estimation of Secretion and Reuptake of Adrenergic Transmitter in the Rabbit Heart

By

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When perfusion methods are used for quantitative estimation of the amount of noradrenaline (NA) secreted from sympathetic neurons it is necessary to prevent trapping of the NA liberated in the tissue, probably mainly in the nerves themselves (cf Iversen 1967) To prevent rebinding of NA supraphysiological frequencies of nerve stimulation have been used, or alternatively drugs known to block neuronal uptake of exogenous amines and in addition high perfusion flow rates have been maintained by various methods Most estimates have been based on experiments with cat spleen The drug most frequently used is phenoxybenzamine (PBA) which in addition to blocking the α-receptors and thus vasoconstriction also at higher concentrations, inhibits uptake of NA from the tissue fluids into the sympathetic neurons (Thoenen et al 1964) In the presence of this drug nerve stimulation at frequencies of 10/sec or less causes a strong increase in the NA efflux from the stimulated tissue The estimates of the amount of NA liberated by each nerve impulse obtained by using this technique in some experiments approach those found when rebinding of NA is minimized by using high frequencies of nerve stimulation (Brown 1965) The agreement between values obtained by these 'pharmacological' and 'physiological' methods suggests that the estimates arrived at may give a reasonably good approximation of the true amounts of NA secreted from the nerves

However the effect on nerve stimulation-induced NA outflow obtained with drugs such as cocaine or imipramine which are generally regarded to be much more efficient than PBA as inhibitors of neuronal uptake of NA have been inconsistent varying from no effect to moderate increase (for a review see Iversen 1967) In view of this discrepancy the present experiments were carried out to compare the effect of optimal concentrations of PBA with that of various uptake inhibitors on nerve stimulation induced NA outflow from the isolated perfused sympathetically innervated rabbit heart, which has the advantage over the spleen that sympathetic nerve stimulation does not cause vasoconstriction The present report includes the results obtained with PBA, 3,3-dimethyl 1 (3-methylaminopropyl)-1-phenyl phthalane (LU 3-010, H Lundbeck & Co A/S) and cocaine A more detailed report will be published elsewhere (Wennmalm to be published)

Forty-nine rabbits weighing from 1 2 to 2 4 kg, were used for the study The rabbits were killed with a blow on the head and the hearts were dissected out with intact sympathetic nerve supply and perfused according to the Langendorff technique (Loffelholz and Muscholl 1969) Heart rate and contractile force were recorded with conventional Grass Equipment The sympathetic nerves to the heart were stimulated with 300 shocks of supramaximal intensity, delivered at 10/sec Each heart was stimulated twice with an interval of 15 min The second stimulation was performed 10 min after the addition of drug(s) to the perfusion medium The perfusate from the heart was collected from the beginning of the stimulation until the mechanical response had faded out, usually for a period of 3—5 min The NA in the perfusate was adsorbed on alumina and analyzed fluorimetrically

In the presence of PBA (3 10^{-6}—3 10^{-5}) LU 3—010 (10^{-8}—10^{-7} M) or cocaine (10^{-5} M), at optimal concentrations, the chronotropic response to nerve stimulation was distinctly prolonged usually three- to fourfold, while its amplitude remained unchanged The inotropic response was largely unaffected by the drugs mentioned The nerve stimulation induced outflow of NA was 216±26 ng (mean±S E M) during the first stimulation This outflow was markedly increased in the presence of the drugs (Fig) At optimal concentrations of PBA or LU 3-010 the NA efflux was slightly more than three times higher than that during the preceding control stimulation, in the abscence of drugs, while a second nerve stimulation in control experiments resulted in an outflow of NA slightly lower (90±12 per cent of control, mean ±S E M) than that seen during the first stimulation period Addition of α- or β blocking drugs like dihydroergotoxine mesylate (0 3 μg/ml Hydergin Sandoz) PBA (3 10^{-6}—3 10^{-5} M) or pronethalol (1 2 10^{-6} M) to the perfusion fluid together with LU 3 010 did not significantly change the effect of LU 3 010 alone on outflow of NA

Cocaine was less efficient in increasing nerve stimulation induced outflow of NA (ratio to control stimulation 2 33±0 07 mean±S E M) In the case of cocaine addition of Hydergin (0 3 μg/ml) potentiated the effect on outflow of NA of cocaine alone to 2 95±0 59

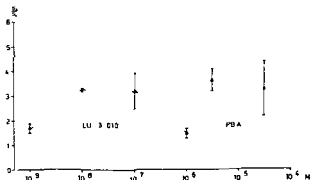


Fig 1 Perfused rabbit heart Ratio (mean±S E M) between outflow of NA during second (S_2 drug added) and first (S_1) nerve stimulation (300 shocks at 10/sec)

The present results which are supported by observations with other uptake inhibitors such as desipramine and protriptyline (Wennmalm, to be published), indicate that even at optimal concentrations these drugs differ in potency to increase nerve stimulation induced outflow of NA This difference can be explained either by assuming that drugs such as cocaine do not completely block the reuptake of NA liberated or that drugs such as PBA or LU 3 010 in addition to blocking the reuptake of NA also increase the amount of NA released The combined effect of cocaine and Hydergin approaches that of PBA or LU 3 010 From the present data it is not possible to judge whether PBA or LU 3 010 on the one hand or cocaine on the other is the drug of choice to block NA reuptake and thus allow a study of the true secretion of NA from the neurons

The present data show that drugs which differ widely chemically at optimal concentrations increase the NA outflow from the tissue in response to nerve stimulation to a relatively narrow interval (2 3—3 7 fold) This suggests that under normal conditions when the uptake mechanism is not depressed more than one half of the amount of NA secreted is recaptured into the neuron. This in itself suggests that recapture of NA secreted may not only be a mechanism for termination of action of the secreted transmitter but by allowing its reuse may also be important from the point of view of transmitter economy (Stjarne 1964)

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Metabolism of ¹⁴C-Histamine in Domestic Animals

II. Cow and Sheep

By

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Abstract

Eliassen, K A *Metabolism of ¹⁴C histamine in domestic animals II Cow and sheep* Acta physiol scand 1971 *81* 289—299

hat for 1 4 methylimidazoleacetic acid

The catabolism of ¹⁴C histamine injected intravenously in goats has previously been examined (Eliassen 1969) In this species oxidative deamination was found to be the only pathway of quantitative importance for the degradation of injected histamine The present study was undertaken to examine whether the metabolic pattern of ¹⁴C histamine found in goats is characteristic for ruminating species

Abbreviations

Hi histamine 4(5))β aminoethyl)imidazole
ethyl imidazole 1 4 MeHi 1 4 methylhistamine
histaminol 4 imidazoyl-etane-2-ol ImAA imida
1 4 methylimidazoleacetic acid 1 methyl imidazo
imidazoleacetic acid 1 methyl imidazole 5 acetic acid ImAA R imidazoleacetic
1 ribosylimidazole-4 acetic acid

Materials and Methods

Thixotropic Gel Powder (Cab O Sil)® was purchased from Packard Instruments (GmbH) Frankfurt am Main Germany Other reagents were the same as in an earl er study (Eliassen 1969) The specific activity of the ring $^{14}C_2$ histamine dihydrochloride used in the present study was 30 5 mCi/mmole

Animals and feeding

One male (75 kg) and three female (55—65 kg) sheep of the Dala breed and two cows of Norwegian red cattle breed (NRF 560 and 500 kg) were used for the ^{14}C histamine experiments Further the histamine concentration in the milk was estimated for 5 other cows of the NRF breed each weighing about 500 kg The cows used in isotope experiments were fed hay whereas the sheep in addition were given kohlrabi and pelleted concentrates Water was allowed *ad libitum* The cows used only for milk sampling were in addition to hay fed concentrates and silage

The sheep were housed in metabolism cages which permitted separate collection of urine and feces The cows urine were obtained by catheterization and collected in plastic (PVC) bottles containing sufficient hydrochloric acid to bring the pH below 2

Injection of ^{14}C labelled histamine

Radioactive histamine (spec act 30 5 mCi/mmole) was purified on a Dowex 50 W column and prepared for injection as previously described (Eliassen 1969) 25 and 100 μCi were injected into the jugular vein of sheep or cows respectively

Biological and chemical determination of histamine and histamine metabolites in urine

Free histamine Urinary free histamine was determined as previously described (Eliassen and Sjaastad 1968) This method involves ion exchange chromatography (Amberlite CG 50) followed by bioassay on isolated guinea pig ileum

Conjugated histamine Conjugated histamine was determined as the increase in free histamine which occurred upon hydrolysis under reflux of acetone treated urine with 10 N HCl for 1 1/2 h

Methylimida oleacetic acid The 1 4 and 1 5 MeImAA were determined according to Granerus and Magnusson (1965) and Granerus (1968 a) The recovery of reference 1 4 MeImAA added to urine was 87 ± 21 % (S D (33 expts) Recovery experiments with 1 5 MeImAA were not performed since reference 1 5 MeImAA was not available in sufficient quantities at the time

Determination of free histamine in milk

Fifty ml of milk was mixed immediately after collection with trichloroacetic acid to a concentration of 5 % After about 1 hr the mixture was centrifuged at 5000 × g for 20 min The supernatant was neutralized with N NaOH and diluted with 1 5 volume of 0 02 M phosphate buffer pH 7 5 20 ml of this mixture was transferred to a Dowex 1 × 8 column (1 2 × 10 cm) in acetate hydroxide form (Kremzer and Wilson 1961) The column was washed with 20 ml 0 02 M phosphate
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and Cohn (1959)

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samples whereas
non radioactive Hi in 10 milk samples was 82 % ± 12 % (S D [illegible]
5 % (S D) Recovery measured by means of ^{14}C Hi seemed therefore useful and the values for milk Hi was corrected on the basis of ^{14}C recorvery measurements The amounts of ^{14}C Hi (0 01 μCi) used for recovery measurements do not significantly contribute to the levels of histamine found in these experiments

Determination of radioactivity in urine feces and milk

Urine 10 to 100 μl urine were plated for counting in a Beckman Lowbeta II flow counter (background 2 1—3 5 cpm) Self absorption was measured by counting urines to which internal standards of ^{14}C Hi had been added

Feces Extraction of feces with hydrochloric acid followed by counting of radioactivity in a

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Carb Spectrometer

Milk 500 μl untreated milk was plated on planchets for counting on a flowcounter in the same way as for urine (Eliassen 1969)

All counts were corrected for self absorption and background activity

Determination of radioactive histamine compounds

urine aliquots and its ethylester was formed and extracted as described by (Granerus and Magnusson 1965) The 1 4 MeImAA ethylester picrate was isolated and after recrystallization the ester picrate was hydrolyzed whereupon the 1 4 MeImAA picrate was formed The picrate of 1 4 MeImAA was then recrystallized until constant activity

Results

Histamine compounds in urine

Free and conjugated Hi Of all urine samples collected from the two cows given ^{14}C histamine, histamine like activity was detectable in only one of the samples The reason for this might be the fact that antihistamine like activity was present in the urine No antihistamine like activity was observed in hydrolyzed urine The total Hi activity (conjugated and possibly some free) of the hydrolyzed urine from the two examined cows is shown in Table I The Hi values are expressed as Hi base and they are corrected for recovery which was very low (50 and 37 % for cow 1 and 2 respectively) compared with that for goat (Eliassen 1969) For all histamine determinations in urine duplicate samples were used

When calculating the urinary excretion of total (free + conjugated) Hi the values found were corrected for the part originating from the injected ^{14}C Hi For cow on an average 4 2 % of the injected ^{14}C Hi was excreted in the urine as free and conjugated Hi (total Hi) which corresponds to about 13 μg

1 4 MeImAA The quantities of urinary 1 4 and 1 5 MeImAA are shown in Table I The values for 1 4 MeImAA are corrected for analytical losses but no correction was made for the part which originated from the injected ^{14}C Hi This part corresponds to less than 0 5 % of the 1 4 MeImAA found

The urinary excretion of free Hi conjugated Hi, and 1 4 MeImAA in sheep has previously been examined in our laboratory (Sjaastad 1967) and is of the order of 4—34 μg 17—68 μg and 0—1 6 mg per 24 hrs, respectively

TABLE I Urinary excretion of total (free and conjugated) Hi 1 4 MeImAA and 1 5 MeImAA in two cows

Cow no	1 4 MeImAA mg/24 hrs	1 5 MeImAA mg/24 hrs	Total Hi µg/24 hrs	moles 1 4 MeImAA / moles total H	^{14}C-1 4 MeImAA / ^{14}C-total H
1	16 4	8 3	110	117	4 9
2	17 2	10 5	195	70	3 7

All values are expressed as base or acid With the exception of 1 5 MeImAA the values are corrected for procedural losses

Free histamine in milk 10 milk samples from 5 cows were analyzed for free Hi The mean histamine concentration determined by biological assay was 0 27 ± 0 18 (S D) µg Hi base/ml milk The fluorimetric method gave up to 2 5 times higher values than the bioassay

Excretion of radioactivity after i v injection of ^{14}C Hi

Urine In Fig 1 a and b is shown the radioactivity in the urine of cows and sheep injected i v with ^{14}C Hi *Most of the injected radioactivity was excreted in the* urine the first 24 hrs after injection In the 4 sheep examined 74 87 75 and 84 % *of the injected radioactivity* were excreted with the urine within the first week The *corresponding figures* for the two cows examined were 99 and 93 % Sheep no 3 did not urinate between 6 and 12 hrs after injection

Feces About 1 % of the injected radioactivity was excreted with the feces during the first week Large amounts of feces particularly in the cows rendered it difficult to measure accurately the low fecal radioactivity Most of the radioactivity was excreted the first and second day after injection but radioactivity above background was registered even on the 7th day

Milk Only about 0 03 % of the injected radioactivity was recovered in milk during the first 24 hrs after injection

Quantitation of ^{14}C Hi metabolites in the urine

In the urine of goats the presence of a radioactive compound assumed to be 4(5 imidazoyl etane 2-ol histaminol has been demonstrated (Eliassen 1969) By means of paper chromatographic technique histaminol was in the present study found to account for about 2 % of the radioactivity in the urine of both cows and sheep

The relationship between time and the urinary excretion of the different Hi metabolites is shown in Table II It is evident that as in the experiments with goats (Eliassen 1969) the ratio between ImAA R and ImAA increased with time

The results of the isotope dilution analyses are shown in Table III The radioactivity of the crystals of the histamine metabolites remained constant after the same number of recrystallizations as previously found in goats (Eliassen 1969) As in goats constant activity of the 1 4 MeImAA picrate was not obtained when the

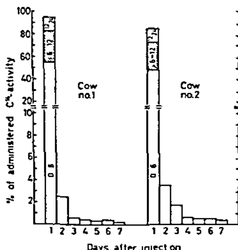


Fig 1 a Urinary excretion of radioactivity the first week after i v injection of ^{14}C labelled histamine to cow The columns for the first day after injection indicate also the excretion of radioactivity between 0—6, 6—12 and 12—24 hrs

isotope dilution was carried out conventionally, but radioautography of paper chromatograms disclosed a spot corresponding to 1 4-MeImAA The presence of ^{14}C-1 4 MeImAA was verified by the modified isotope dilution technique developed for this metabolite (see above) Only one 24 hr urine from cow and sheep was examined by this modified technique (Table III)

One goat (goat C, Eliassen 1969) was also examined by the modified isotope dilution technique for 1 4-MeImAA 1 4-MeImAA was found to account for 15 % of the urinary radioactivity, whereas the corresponding figure found by paper chromatography was 12 % (Eliassen 1969) The figures obtained with the isotope di-

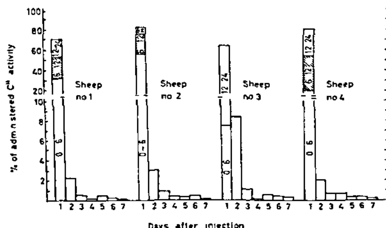


Fig 1 b Urinary excretion of radioactivity the first week after i v injection of ^{14}C-labelled histamine to sheep The columns for the first day after injection indicate also the excretion of radioactivity between 0—6 6—12 and 12—24 hrs

TABLE II Histamine metabolites in urine specimens collected at different intervals of time in sheep no I after i v injection of ^{14}C-histamine The values are given as % of excreted radioactivity

Paper chromatographic determination					
	Isotope dilution determination				
	0—6 hrs	6—12 hrs	12—24 hrs	sum 24 hrs	24 hrs
Hi	3 4	0 4	0 5	0 5	2 3
MeHi	1 1	0 2	—	0 3	1 0
HiOH	0 8	0 4	0 1	0 5	—
ImAA	22 5	12 0	8 5	11 7	20
ImAA R	39 0	61 4	69 3	60 8	42
MeImAA	31 1	23 4	17 7	23 8	—
Appl point	2 0	2 2	3 9	2 5	—
Sum	100 1	100 0	100 1	99 9	—

lution technique were somewhat higher than those found with paper chromatography

Conjugated Hi normally present in urine is believed to be identical with AcHi (Tabor and Mosettig 1949) Paper chromatography of urine showed that if any of the injected ^{14}C-Hi was excreted as ^{14}C AcHi, the quantities must be insignificant. It is however, possible that the injected ^{14}C Hi could be excreted in other conjugated forms than AcHi If so, the present analyses show that such conjugates would account for less than 0 7 % of the radioactivity in the urine

TABLE III Quantitative analysis for histamine metabolites in the urine of sheep and cow after

Animal	Excreted radioactivity per 24 hrs in % of injected	Histamine Metabolites % of total ^{14}C in the first 24 hrs urine		
		Histamine	1,4 Methyl histamine	Imidazoleacetic Free
Sheep I*	70	2 3 (0 5)	1 0 (0 3)	20 (12)
Sheep II	82	4 5 (5 0)	1 7 (1 0)	20 (20)
Sheep III	64	3 2 (1 5)	0 7 (0 8)	17 (18)
Sheep IV	81	0 9 (0 5)	0 3 (0 6)	16 (14)
Mean	74	2 8 (1 9)	0 9 (0 7)	18 (16)
Cow I	95	3 7 (3 3)	0 7 (1 1)	17 (18)
Cow II	86	4 6 (2 9	0 7 (1 0)	19 (18)
Mean	90	4 2 (3 1)	0 7 (1 0)	18 (18)

* s c injection
( ) Determined by means of paper chromatography Values are not corrected for incomplete recovery from chromatograms.
· Determined by the isotope dilution technique which includes esterification and extraction

Discussion

The metabolic patterns of ^{14}C Hi in cows and sheep are very similar to that found in goats (Eliassen 1969) Compared with species previously examined oxidative deamination seems to be of great importance for Hi degradation in ruminants Of other species so far examined only the rat exhibits oxidative deamination of about the same order of magnitude. From the present experiments and from the experiments with goats (Eliassen 1969) oxidation of Hi to imidazoleacetaldehyde followed by a reduction to HiOH appears to be a consistent but minor pathway for Hi degradation in ruminants

The use of i v injection of ^{14}C Hi as a tool in the evaluation of the catabolism of endogenous Hi has been a subject of controversy However, the metabolism of parenterally administered ^{14}C-Hi may at least give valuable information on comparative aspects of Hi metabolism It is likely that the metabolic pattern for injected ^{14}C Hi is not identical in all excreta Valid conclusions with regard to the metabolism of injected ^{14}C Hi from analyses of its metabolites in the urine therefore necessitates that most of the injected radioactivity is excreted by this route In the present experiments this prerequisite seemed to be met since about 74 and 90 % of the injected radioactivity was recovered in the urine of sheep and cows, respectively

The fraction of the injected radioactivity recovered in the urine of sheep was of the same order of magnitude as previously found in goats (Eliassen 1969) The corresponding figure in cows was somewhat higher and this could be explained neither by differences in fecal excretion of radioactivity nor by inferiorities in the technique used for collection of urine The recovery of radioactivity in urine in other species

injecting ^{14}C histamine

acid		1 4-Methyl imidazole acetic acid	Conjug hista mine	Hista minol	Sum of meta bolites
Ribos de	Total				
42 (61)	62 (73)	(24)	<0 7	(0 5)	90 5
41 (49)	61 (69)	(22)	<0 7	(1 9)	91 1
42 (59)	59 (77)	25 (18)	<0 7	(3 0)	83 9
56 (65)	72 (79)	(16)	<0 7	(1 6)	90 8
46 (58)	64 (74)	(20)	<0 7	(1 8)	89 5
56 (59)	73 (77)	(18)	<0 7	1	96 4
52 (58)	71 (76)	20 (17)	<0 7	4	97 3
54 (58)	72 (76)	(18)	<0 7	2	96 9

Mean values for urinary excretion of total radioactivity are expressed as per cent of the amount injected Unchanged ^{14}C histamine and its main metabolites are expressed as per cent of the urinary excreted total radioactivity

after injection of ^{14}C-Hi has been more variable, but is generally of the same range as in ruminants

The radioactivity which could not be accounted for in urine and feces was presumably retained in the body or excreted by other routes. Experiments with injection of radioactive Hi in rats (Schayer 1952, Snyder, Axelrod and Bauer 1964, Beaven *et al.* 1968 and Johnson 1970) showed that part of the radioactivity was very rapidly absorbed in the tissues but also relatively rapidly released. Only very small amounts of radioactivity persist in the tissues after 48 hrs. Since the available evidence does not support the idea that administered Hi is retained to any extent in the body for a long period of time, other ways of excreting ^{14}C-Hi and its metabolites than elimination with urine and feces might exist. After administration of ^{14}C-Hi into rumen of sheep by way of a rumen cannula Sjaastad and Kay (to be published) showed that much of the radioactivity was exhaled as $^{14}CO_2$. Furthermore after s.c. injection of ^{14}C-Hi to sheep Sjaastad (1967 a) found radioactivity in the rumen liquor. It is thus possible that some of the radioactivity of the injected ^{14}C-Hi was exhaled as $^{14}CO_2$. If so, this mechanism for the degradation of parenterally administered ^{14}C-Hi might be confirmed to ruminants. Thus Schayer found that rats injected with ring 2-^{14}C Hi exhaled only a minute amount of $^{14}CO_2$, which according to the author may have originated from an impurity in the ^{14}C-Hi (Schayer 1952).

The Hi metabolites determined by the isotope dilution technique did not account for all of the urinary radioactivity. By counting the area on the chromatograms which produce spots on the X-ray films, it is possible to account for about all of the radioactivity in the urine. The difference in results by isotope dilution and by paper chromatographic techniques in quantitating the Hi metabolites is probably limited to the figures for ImAA-R. For the other metabolites the two methods give about the same results (Table III). The inexpensive paper chromatographic method therefore seems useful for the semiquantitative determination of ^{14}C-Hi metabolites in urine.

Both for cows and sheep the counts obtained by the isotope dilution technique for total ^{14}C-Hi (free + conjugated) in urine were not significantly different (0.7 % of total urinary radioactivity) from those found for free ^{14}C-Hi. This is in accordance with the findings that paper chromatography of urine did not reveal detectable radioactivity (less than 0.5 % of urinary radioactivity) associated with carrier AcHi on the chromatograms. Experiments done by Sjaastad (1967 b) indicate that some form of conjugation of Hi takes place in sheep tissues *in vitro*. He found that 0.9 ± 0.5 % of non labelled Hi added to sheep liver homogenates or slices were conjugated when incubated for 1 1/2 hour. In the present study, when kidney and liver homogenates from goat and sheep were incubated with ^{14}C-Hi and X-ray films were exposed to chromatograms of the incubation mixtures (Eliassen 1969 and Eliassen unpublished) less than 0.2 % of total radioactivity could be accounted for in the AcHi area on the chromatograms. This might be due to differences in the sensitivity between the non-isotopic method used by Sjaastad and the isotopic method used in the present experiment, or to the existence of forms of conjugated Hi other than AcHi.

In the present experiments it was not possible to estimate free Hi in the urine of cows by bioassay the explanation probably being the presence of antihistamine like activity in the urine Bolotti (1951) has found by bioassay that the Hi content in cow urine varied from non detectable amounts to 0 02 μg/ml (mean 0 008 μg/ml)

It is generally accepted that urinary 1 4 MeImAA is a metabolite of histamine only It is therefore noteworthy that the specific radioactivity of total Hi is up to 24 times larger than that for 1 4 MeImAA The large difference between specific radioactivities of the two metabolites might have been even larger if free Hi instead of total Hi (free + conjugated) could be used for the calculations Provided that endogenous and injected Hi are catabolized in the same way this indicates that most of 1 4 MeImAA in the urine is formed before it reaches the circulating system A difference in specific radioactivities of Hi and 1 4 MeImAA has also been found in horses and pigs (Eliassen 1970) Also the ratios 1 4 MeHi/Hi and 1 4 MeImAA/Hi calculated from experiments in man is greater for the non radioactivity than for the radioactive compounds after parenteral (Lindell *et al* 1960 Fram and Green 1965 and Granerus Wetterquist and White 1968) or oral administration (Granerus 1968 b) of ^{14}C Hi Granerus *et al* (1968) expressed the opinion that results from their experiments with man and those of Lindell *et al* (1960) might indicate that intravenously injected Hi is methylated less efficiently than histamine released within the tissue It seems possible moreover that not only may the Hi released within the cell but also Hi supplied within the food be more efficiently methylated than intravenously injected Hi Granerus (1968 b) found that diets with varying protein content caused a great variation in urinary Hi and its methylated metabolites In the dog however Hi or histidine absorbed from the intestine do not seem to contribute to the next 24 hour excretion of 1 4 MeImAA (Tham 1966) In rat urine similar specific radioactivity for Hi and 1 4 MeHi has been observed some days after s c injection of small daily doses of ^{14}C histidine had been discontinued (Wetterquist and White 1968)

The results in Table II demonstrate that most of the unmetabolized ^{14}C-Hi is excreted during the first hours after the injection These observations are in accordance with those of Adams Hardwick and Spencer (1957 who found that Hi injected intravenously in the cat is removed from the blood within a few minutes as well as the findings of Arnoldson *et al* (1962) that the renal extraction of Hi is about 80 % for each passage of the blood The observations indicate that a single injection of ^{14}C Hi possibly may give too high specific radioactivity for Hi compared with the Hi metabolites It is therefore probable that the specific radioactivity of Hi and its metabolites in urine after injection of ^{14}C-Hi would be more similar if the urine excreted during the first minutes after injection were discarded

It appears from the paper chromatograms (Table II) that the ratio ImAA R/ImAA in urine increases with time as in the experiments with goats (Eliassen 1969) Corresponding results for the two metabolites have been reported for rat tissue Snyder *et al* 1964 Johnson 1970) The increasing ratio may be explained by

ferences in the rates of formation as well as rates of elimination of the two compounds

The high levels of 1 5-MeImAA found in cows' urine, using the method of Granerus (1968 a), are in accordance with the results obtained for sheep by Sjaastad (1967 a) He found that 1 5-MeImAA was a component of the urine even when 1 4-MeImAA could not be detected In this study no radioactivity could be detected in the 1 5-MeImAA spot This supports the assumption that 1.5-MeImAA is not formed in the body tissues or fluids

High levels of Hi has been found in cows' milk (Bolotti 1951, Wrenn et al 1963, Zarkower, Dunlop and Norcross 1965, Zarkower 1967) These results were confirmed in the present experiments However, only small amounts (0 03 %) of the injected radioactivity could be accounted for in the milk Since as much as about 4 % of the injected radioactivity was excreted in the urine which, on the other hand had a low histamine concentration it seems unlikely that histamine in the milk is directly derived from the blood

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Metabolism of ^{14}C-Histamine in Domestic Animals

III. Horse and Pig

By

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Abstract

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The metabolism of ^{14}C histamine injected i.v. in 3 ruminant species (goat, sheep and cow) has been studied previously. In the present study isotope dilution and paper chromatographic techniques were used to examine urinary ^{14}C metabolites of i.v. injected ^{14}C histamine in the horse and the pig. The urine excreted the first 24 hrs after injection accounted for 64—76 % and 81—91 % of the radioactivity injected in horses and pigs, respectively. Less than 2 % of the injected radioactivity could be accounted for in the feces excreted the first week after injection. Known metabolites in the first 24 hr urine on an average accounted for about 94 % and 103 % of the urinary radioactivity in horses and pigs respectively. Methylation of histamine to 1,4-methylhistamine and further oxidation to 1,4-methylimidazoleacetic acid was quantitatively the most important pathway for histamine degradation, accounting on an average for about 63 % and 76 % of the radioactivity in horse and pig urine, respectively. The horse and the pig seemed to metabolize histamine in quite a similar way which again was quite different from that previously found in the ruminants in which oxidative deamination of histamine to imidazoleacetic acid was the only pathway of quantitative importance. As in ruminants histaminol seemed to be a metabolite of i.v. injected histamine accounting for about 2 % of the urinary radioactivity. The specific radioactivity of histamine was about tenfold larger than for 1,4-methylimidazoleacetic acid in horses and pigs.

The metabolism of i.v. injected ^{14}C histamine in goats, sheep and cows has previously been studied (Eliassen 1969 and 1971). Oxidative deamination to imidazoleacetic acid partly followed by conjugation with ribose was the main pathway for degradation of histamine in the three ruminating species mentioned. Methylation of histamine was of minor importance.

Abbreviations

Hi histamine 4(5))β aminoethyl)imidazole

1,4-MeH 1,4-methylhistamine

da

zole-4-acetic acid

acid ImAAR imidazoleacetic acid riboside

1 ribosylimidazole

It was considered of interest also to examine the catabolism of ^{14}C histamine in horses and pigs Studies of histamine inactivation in horse and pig have been re stricted to in vitro experiments on the oxidation of histamine in blood serum of both species (Kolb 1956) and in the mucosa of horse stomach (Kolb 1958)

Materials and Methods

Norwegian "Land svin breed (60 140 and
were used One of the horses was of the
ed (580 kg) and one halfbreed (500 kg)
permitted separate collection of urine and
feces whereas urine of the horses was collected by means of bags fitted to the genitalia Suf
lection bottles
ses were fed hay and oats
in collaboration with Maj Britt Johansson, Clinical Physiological Laboratory Lasarettet Lund Sweden Histamine (2 ring ^{14}C) spec act 54 mCi/mmole was obtained from the Radiochemical Centre Amersham England Otherwise the methods and reagents in this study were those described previously (Eliassen 1971)

Injection of ^{14}C labelled histamine The purity of the injected ^{14}C Hi was checked by two-dimensional paper chromatography followed by autoradiography Only one spot was detected The ^{14}C-Hi was dissolved in saline and injected into the jugular vein without further purifi cation The horses were given about 100 μCi whereas the pigs were given about 25 μCi One μCi corresponds to 2 06 μg Hi base

Results

Urinary excretion of Hi and 1 4 and 1 5 MeImAA

Free Hi Free histamine was determined in urine collected the first 24 hrs after injection The values in Table I are given in terms of the base and they are corrected for analytical losses The recovery of histamine diphosphate (10—100 μg/100 ml 10 expts) was 78 6 $\pm$ 7 2 % (S D) for both species

In horses approximately 6 μg corresponding to about 15 % of the biologically active Hi could originate from the injected ^{14}C Hi since on an average about 3 % of

TABLE I Urinary excretion of Hi 1 4 MeImAA and 1 5 MeImAA in horse and pig

Animal	1 4 MeImAA mg 24 hrs	1 5 MeImAA mg/24 hrs	Free Hi μg 24 hrs	moles 1 4 MeImAA / moles Hi	^{14}C 1 4 MeImAA / ^{14}C Hi
Horse 1	13 9	2 5	43	256	15 1
Horse 2	13 3	1 5	46	229	22 7
Horse 3	20 1	1 3	39	409	26 4
Mean	15 8	1 7	43	298	21 4
Pig 1	65 4	0	230	226	19 0
Pig 2	83 5	0	200	331	24 6
Pig 3	79 5	0	370	169	23 5
Mean	76 1	0	267	242	22 4

All values are expressed as base or acid
With exception of 1 5 MeImAA the values are corrected for procedural losses

TABLE II Quantitative analyses for histamine metabolites in the urine of horse and pig after in-

Animal	Excreted radioactivity per 24 hrs in % of injected	Histamine Metabolites, % of Total ^{14}C in the first 24 hrs		
		Histamine	1 4 Methyl-histamine	Imidazole acetic
				Free
Horse I	64	3 9 (3)	3 6 (2)	0 7
Horse II	73	2 6	4 4	0 5
Horse III	76	2 2	3 8	1 4
Mean	71	2 9	3 9	0 9
Pig I	85	3 3 (4)	12 4 (10)	8 (8)
Pig II	94	2 6	11 7	11 (12)
Pig III	81	2 9 (2)	10 2 (8)	1 (3)
Mean	87	2 9 (3)	11 4 (9)	7 (7)

( ) Determined by means of paperchromatography Values are not corrected for incomplete recovery from chromatograms

the injected ^{14}C-Hi was excreted unchanged The values for free Hi in horse urine were therefore corrected on this basis (3 %) In pigs only an insignificant fraction of urinary free Hi could originate from the injected ^{14}C Hi and no correction was made

1 4- and 1 5-MeImAA The values for urinary MeImAA the first 24 hrs after injection of ^{14}C-Hi are given in Table I The recovery for 1 4-MeImAA hydro

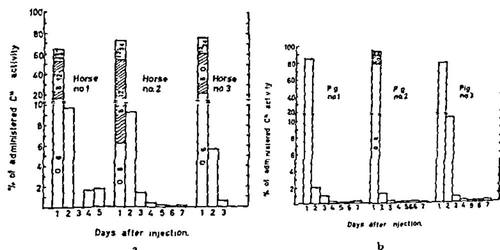


Fig 1 a. Urinary excretion of radioactivity the first week after i v injection of ^{14}C labelled histamine in horse The columns for the first day after injection indicate also the excretion of radioactivity between 0—6 6—12 and 12—24 hours

Fig 1 b Urinary excretion of radioactivity the first week after i v injection of ^{14}C labelled histamine in pig For pig no 2 the column for the first day after injection indicates the excretion of radioactivity between 0—6, 6—12 and 12—24 hours

jecting ^{14}C-histamine

urine					
acid		1 4 Methyl imidazole acetic acid	Conjugated histamine	Histaminol	Sum of metabolites
Riboside	Total				
19	20 (27)	59 (65)	<1	(2)	89
23	24	59	<1	(1)	91
34	35	58	<1	(2)	101
25	26	59	<1	(2)	94
16 (20)	24 (27)	63 (59)	<0 8	(2)	104
12 (22)	23 (35)	64 (52)	<0 8	(1)	102
18 (23)	19 (26)	68 (58)	<0 8	(1)	101
15 (22)	22 (26)	65 (56)	<0 8	(1)	103

Mean values for urinary excretion of total radioactivity are expressed as per cent of the amount injected Unchanged ^{14}C-histamine and its main metabolites are expressed as per cent of the ' total radioactivity in urine

chloride added to urine (7 expts) was 70 ± 10 % (S D) and the values were corrected on this basis Further the values for 1 4 MeImAA were also corrected for 1 5 MeImAA originating from the injected ^{14}C-Hi On an average this corresponds to 200 and 55 µg of 1 4-MeImAA in pigs and horses, respectively The values for 1 4-MeImAA were not corrected for analytical losses since this substance was not available in quantities sufficient for recovery experiments

Radioactivity in urine and feces after i v injection of ^{14}C-Hi

Urine Fig 1 a and 1 b demonstrate the radioactivity in the urine the first days after i v injection of ^{14}C Hi Most of the radioactivity was recovered in the urine excreted the first 24 hrs after injection The recovery of the radioactivity for the

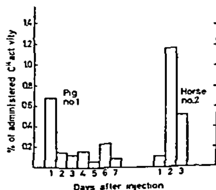


Fig 2 Fecal excretion of radioactivity the first days after i v injection of ^{14}C-labelled histamine

TABLE III *Histamine metabolites in urine specimens collected at different intervals of time in horse no 1, after i v injection of* [14]*C-histamine The values are given as % of excreted radioactivity*

	Paper chromatographic determination				Isotope dilution determination
	0—6 hrs	6—12 hrs	12—24 hrs	sum 24 hrs	24 hrs
Hi	7.9	0.6	0.7	2.5	3.9
MeHi	3.3	2.0	1.2	2.2	3.6
HiOH	1.8	1.3	0.6	1.3	—
ImAA	2.5	{ 23.6	6.6	{ 26.7	0.7
ImAA R	12.1		48.4		19.0
MeImAA	69.7	70.5	41.4	65.0	59.0
Appl point	2.6	1.6	1.2	1.8	—
Sum	99.6	99.6	100.1	99.5	89.2

first week was 88, 96 and 93 % for pigs no 1 2 and 3, respectively In horse no 2 the corresponding value was 84 % In horses no 1 and 3 the urine was examined only for the first 5 and 3 days after injection respectively The recoveries were 77 and 84 %

Feces The figures for fecal radioactivity in pig no 1 and horse no 2 are shown in Fig 2 For the remaining animals feces were not systematically analysed Roughly the analyses showed that less than 2 % of the injected radioactivity is excreted with feces the first week after injection

Estimation of [14]*C histamine metabolites in the urine*

Isotope dilution technique as well as paper chromatograph was used for identification and quantification of [14]C Hi metabolites (Table II) Constant radioactivity of the crystals of Hi and all Hi metabolites was obtained at a point between the 2nd and the 7th crystallization Since HiOH was not available in sufficient quantities to allow estimation by isotope dilution technique HiOH was only estimated by counting of the spot corresponding to HiOH on the paper chromatograms Accurate estimation of urinary metabolites of Hi especially HiOH by paper chromatography was difficult probably because the low specific radioactivity of the urine specimens The *HiOH spot on the paper chromatograms seemed however* to account roughly for about 2 % of the injected radioactivity The paper chromatograms also provided evidence that *if* [14]*C AcHi was excreted subsequent to injection* of [14]C-Hi the quantities are less than 1 % of the injected radioactivity This was confirmed by isotope dilution technique

The relationship between time and the urinary excretion of the different Hi metabolites in horse no 1 is shown in Table III It is evident that most of the unmetabolized Hi is excreted the first 6 hrs after injection

Discussion

The metabolic patterns of ^{14}C Hi in horses and pigs were surprisingly similar and deviated considerably from those of the ruminants (Eliassen 1969 and 1971) In horses and pigs methylation of Hi followed by oxidation to 1 4-MeImAA seemed to be of the same quantitative importance as the oxidative deamination is in ruminants In this connection it is worthy of mention that Kolb (1956) found considerable oxidation of Hi in horse and pig sera However, this does not necessarily reflect the oxidation of Hi to ImAA, but might be due partly or entirely to the oxidation of 1 4 MeHi

^{14}C-HiOH appeared to be a urinary metabolite of i v injected ^{14}C Hi in horses and pigs but as in ruminants it was of small quantitative importance Further, as in the ruminants horses and pigs seem to metabolize efficiently physiological quantities of Hi that reach the systemic circulation since less than 3 % of the i v injected Hi is excreted unchanged with the urine In horse and pigs the specific radioactivity of Hi was about tenfold larger than the specific radioactivity of 1 4 MeImAA A similar difference between the specific radioactivity of Hi and 1 4-MeImAA was found in experiments with cows (Eliassen 1971), where these findings were discussed

The fraction of injected ^{14}C Hi recovered as radioactivity in the urine the first days after injection was of the same order as that previously found for ruminants (Eliassen 1969 and 1971) Most of the radioactivity was excreted along with the urine during the first 24 hrs after injection Analyses of the Hi metabolites in the first 24 hr urine therefore seems to permit valid conclusions with regard to the overall metabolism of injected ^{14}C Hi In the present experiments paper chromatographic technique was not as suitable for semiquantitation of Hi metabolites in the urine as in experiments with ruminants (Eliassen 1971) This difference may as mentioned earlier be caused by the low specific radioactivity of the horse and pigs urines

At variance with what was found with urine from ruminants (Eliassen 1969 and 1971) constant radioactivity was in the present experiments obtained for the 1 4 MeImAA picrate crystals A different batch of 1 4 MeImAA carrier was used in the previous experiments but control experiments showed that this was not the underlying cause for the difference As in sheep (Sjaastad 1967) and cows (Eliassen 1971) the 1 5 isomer of MeImAA was also found in horse urine but not in pig urine

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Acta physiol. scand. 1971. 81. 307—314

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The Effect of Combined Respiratory and Nonrespiratory Alkalosis on Energy Metabolites and Acid-Base Parameters in the Rat Brain

By

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Abstract

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In order to study the cerebral effects of a combined respiratory and nonrespiratory alkalosis,

The marked reduction in cerebral blood flow during extreme hypocapnia (Kety and Schmidt 1946, Cain 1963, Wollman *et al.* 1965) raises the question whether or not cerebral hypoxia accompanies marked hyperventilation. However, although the accompanying EEG changes (Davis and Wallace 1942, Gotoh *et al.* 1965) and the reduction of the tissue and cerebral venous oxygen tensions (Sugioka and Davis 1960, Cain 1963, Alexander *et al.* 1965, Gotoh *et al.* 1965) indicate the presence of tissue hypoxia the results are far from conclusive. Further doubts were raised by previous attempts to demonstrate an anaerobic production of lactic acid in that both Cain (1963) and Alexander *et al.* (1965) failed to record an increased "excess lactate" production (see Huckabee 1958) by the brain during marked hyperventilation. The decreased aerobic and increased anaerobic utilization of glucose reported by the latter authors cannot be taken as conclusive evidence of hypoxia since it is well known

that alkalosis *per se* increases lactate production in the tissue (Domonkos and Huszák 1959, Leusen and Deemester 1966).

More convincing evidence of the presence of cerebral hypoxia during marked hyperventilation has been presented during the last years. Thus, by reducing the arterial CO_2 tension to 10 mm Hg Alexander *et al.* (1968) observed a 10 % reduction of $CMRO_2$ and a significant elevation of the cerebral venous "excess lactate" level. Further, it has been demonstrated that hyperventilation during the inhalation of 100 % oxygen at three atmospheres absolute pressure abolishes the usual EEG slowing and reduces the lactate production (Reivich *et al.* 1968). Finally, results from our laboratory have shown that marked hyperventilation in cats increases the brain tissue and CSF lactate/pyruvate ratio (Granholm and Siesjö 1969) as well as the tissue NADH level as recorded by surface fluorometry (Granholm *et al.* 1969).

The hypoxic influence of marked hyperventilation is attributable to both reduction in flow and to decreased transport of oxygen due to the Bohr effect (Cain 1963, Gotoh *et al.* 1965). In an attempt to study the latter effect Alexander *et al.* (1968) produced metabolic alkalosis in man when $paCO_2$ was held at 19 mm Hg. The increase in pH did not lead to the expected aggravation in the metabolic changes but to an unexpected rise in cerebral blood flow (Wollman *et al.* 1968).

The present work had two objectives. The first was to re-evaluate the metabolic effects of marked hyperventilation in animals under light nitrous oxide anesthesia by measuring labile organic phosphates as well as all parameters necessary for a calculation of cytoplasmatic NADH/NAD ratios in the brain. The second aim of the study was to evaluate the effect of a superimposed metabolic alkalosis. In order to allow a comparison with the results of Alexander *et al.* (1968) the procedure used for inducing the acid base changes was similar to that used by these authors.

Methods

Male rats of the Wistar strain, weighing 300—350 g, were tracheotomized after the induction of anesthesia with divinyl ether. The animals were then maintained on 70 % N_2O and 30 % O_2 and ventilated with a Starling type respirator (Braun, Melsungen). The rats were immobilized with tubocurarine chloride ("Tubocurarin", Vitrum) i.p. One femoral vein was [illegible] artery was cannulated [illegible] er Elema Stock[illegible] ents and analysed [illegible] meter, Copenhagen [illegible] ere obtained from the alignment nomogram of Siggaard Andersen (1963). The hemoglobin concentrations were photometrically determined (Vitatron, Holland). The body temperature was measured in the rectum with a mercury thermometer. The atlanto-occipital membrane was exposed for later [illegible] of CSF. A longitudinal skin incision was made over the top of the skull and a plastic [illegible] funnel [illegible] hyper[illegible] ned [illegible] degree [illegible] ughout [illegible] a hyper[illegible] body [illegible] ection [illegible] the ex-

periment was terminated During this period, repeated blood samples were drawn for the

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The bicarbonate concentration of the CSF was calculated from the total CO_2 content by subtracting the dissolved CO_2 calculated as the product of the CSF CO_2 tension (Ponten and *Siesjo 1966) and the solubility factor 0 03135 mMoles/kg/mm Hg (Siesjo 1962) CSF pH* was calculated using a pK value of 6 120 (Mitchell *et al* 1965) which is corrected for pH

The intracellular bicarbonate concentration was calculated as

$$(HCO_3^-)_i = \frac{TCO_2 - 0\,0292 \;\; PtCO_2 - 0\,03\,(HCO_3^-)_{bl} - 0\,12\,(HCO_3^-)_{CSF}}{0\,64}$$

lubility factor for (HCO3)bl the nate contained in The correspond te concentration factor of 0 0314 mMoles/mm/kg of i c water The CSF and mean tissue CO_2 tensions were derived from the relation described by Ponten and Siesjo (1966) which was checked in control experiments

experiments in each group

The cytoplasmatic $NADH/NAD^+$ ratios were obtained from the intracellular lactate and pyruvate concentrations, and from the calculated pH_i using the equation

$$\frac{NADH}{NAD^+} = \frac{\text{Lactate}}{\text{Pyruvate}} \cdot \frac{K}{(H^+)}$$

with a K value of 1 11 10 11 (Williamson *et al* 1967) The differences between the series were statistically examined using Wilcoxon s rank sum test

Results

For the calculation of the pH of the extra and intracellular spaces in the present experiments the CSF and the mean tissue CO tensions were obtained by adding 7 mm Hg to measured arterial CO_2 tensions (Ponten and Siesjo 1966) Since the relations described in this publication were obtained in cats four control experiments were performed on rats with measurements of the CO tensions in arterial blood and in cisternal CSF after 45 min of hyperventilation In two of the rats sodium chloride was infused and in these animals the CSF/blood pCO_2 differences were 7 9 and 8 0 mm Hg respectively while the corresponding differences for the two sodium bi carbonate rats were 6 6 and 7 1 mm Hg respectively The results thus were in relatively close agreement with the relations described by Ponten and Siesjo (1966) Moreover they suggested that a ΔpCO of 7 mm Hg could be used for all experiments especially since much larger pCO differences are needed to give significant

changes in pH_i' between the groups injected with sodium chloride and sodium bicarbonate, respectively (see below)

In order to make possible a correction for the blood lactate and pyruvate contents in the calculations of intracellular concentrations, these metabolites were measured in four control experiments In two rats injected with sodium chloride the whole blood lactate concentrations were 4 42 and 4 69 mMoles/kg respectively, while the corresponding pyruvate concentrations were 0 231 and 0 188 mMoles/kg These values are markedly higher than those measured under normocapnic conditions (see Nilsson and Siesjo 1970) When a metabolic alkalosis was added to the respiratory one the values were further increased Thus, when sodium bicarbonate was given the lactate concentrations measured were 7 12 and 7 30 and the pyruvate concentrations 0 265 and 0 210 mMoles/kg respectively

Since the main aim of the present experiments was to compare the effects of respiratory alkalosis with those of combined respiratory and nonrespiratory alkalosis no normocapnic control groups were made However, in order to illustrate the metabolic changes observed in the experiments these have been compared to the values recently published for normocapnic animals using an identical anesthesia and a very similar technique (Nilsson and Siesjo 1970)

(metabolic and respiratory alkalosis) Means ± S E are given The values for the normocapnic control animals have been taken from a previous normocapnic control group obtained during nitrous oxide anaesthesia (Nilsson and Siesjo 1970)

	Arterial blood					CSF					
Experimental group	pH	pCO_2	HCO_3^- calc	pO_2	Hb	TCO_2	HCO_3 calc	pH calc	La	Py	La/py
Control series n = 7	7 40 ±0 01	39 3 ±1 6	24 7 ±0 6	—	15 4 ±0 3	—	—	—	2 98 ±0 17	0 196 ±0 028	16 0 ±1 3
Respiratory alkalosis	7 58	16 5	15 3	125	13 4	21 94	21 2	7 58	6 48	0 263	24 6
	7 63	15 4	15 8	178	14 2	21 81	21 1	7 60	6 76	0 307	22 0
	7 52	16 4	13 9	157	11 5	22 94	22 2	7 60	6 03	0 268	22 5
	7 58	16 3	15 0	171	11 7	25 28	24 6	7 65	5 90	0 224	26 4
	7 58	14 9	13 7	201	13 6	23 31	22 6	7 64	5 50	0 247	22 3
	7 61	15 1	14 8	196	12 8	24 69	24 0	7 66	6 51	0 239	27 2
	7 58 ±0 02	15 8 ±0 3	14 8 ±0 3	171 ±11	12 9 ±0 4	23 33 ±0 58	22 6 ±0 6	7 62 ±0 01	6 20 ±0 19	0 258 ±0 012	24 2 ±0 9
Metabolic and respiratory alkalosis	7 78	17 6	26 2	150	15 3	21 35	20 6	7 55	6 01	0 197	30 5
	7 80	16 0	24 8	156	13 4	23 89	23 2	7 63	6 67	0 262	25 5
	7 81	16 8	26 8	93	13 4	23 81	23 1	7 61	7 40	0 263	28 [illegible]
	7 82	15 2	24 7	170	13 9	24 03	23 3	7 64	7 01	0 279	25 1
	7 85	14 7	25 8	187	14 1	24 84	24 2	7 67	6 25	0 232	26 9
	7 73	16 6	21 9	168	13 8	25 06	24 3	7 64	6 14	0 240	25 6
	7 80 ±0 02	16 2 ±0 4	25 0 ±0 7	154 ±13	14 0 ±0 3	23 83 ±0 54	23 1 ±0 5	7 62 ±0 02	6 58 ±0 22	0 246 ±0 012	27 0 0 8

TABLE II The tissue CO_2 contents and the lactate, pyruvate, ATP, ADP, AMP, and phosphocreatine contents in the rat brain during respiratory and combined metabolic and respiratory alkalosis (*cf* Table I) The contents are given in mmoles/kg of wet tissue Means ± S E The normocapnic values are those published by Nilsson and Siesjö (1970)

Experimental group	TCO_2	La	Py	ATP	ADP	AMP	PCr	La/py
Normocapnia	13 71	1 56	0 098	2 80	0 38	0 02	5 04	16 1
	±0 36	±0 07	±0 005	±0 04	±0 02	±0 01	±0 06	±0 6
Respiratory alkalosis	8 23	4 12	0 195	3 02	0 37	0 02	5 49	21 1
	7 68	4 51	0 204	2 75	0 40	0 03	4 86	22 1
	8 68	3 72	0 180	2 89	0 39	0 04	5 05	20 1
	8 82	3 75	0 186	2 86	0 36	0 03	5 03	20 1
	8 14	4 29	0 186	2 85	0 41	0 02	4 96	23 1
	8 60	4 26	0 196	2 76	0 38	0 03	5 06	21 7
	8 36	4 11	0 191	2 85	0 38	0 03	5 08	21 5
	±0 17	±0 13	±0 004	±0 04	±0 01	±0 01	±0 09	±0 4
Metabolic and respiratory alkalosis	8 95	4 41	0 185	2 84	0 38	0 03	5 01	23 8
	9 36	5 28	0 214	2 82	0 37	0 01	5 09	24 6
	8 81	4 92	0 228	2 84	0 38	0 04	5 13	21 6
	—	4 55	0 213	2 82	0 38	0 03	5 29	21 4
	8 41	6 38	0 232	2 91	0 41	0 04	4 96	27 5
	8 94	5 58	0 217	3 05	0 39	0 03	5 43	25 7
	8 89	5 19	0 215	2 88	0 38	0 03	5 15	24 1
	±0 15	±0 30	±0 008	±0 04	±0 01	±0 01	±0 07	±1 0

Table I shows the blood and CSF parameters for the individual rats compared to the mean values for the normocapnic state There were no apparent differences in the CO_2 or O_2 tensions, or in the hemoglobin concentrations, between the groups injected with sodium chloride and sodium bicarbonate respectively but a marked difference in the plasma pH, and in the plasma bicarbonate concentration ($p < 0\ 001$) There was no significant difference in the mean arterial blood pressure between the sodium chloride and sodium bicarbonate groups since the mean values (± S E) were 135 ± 7 and 130 ± 9 mm Hg respectively In the CSF, there were no differences in the total CO content in the bicarbonate concentration or in the calculated pH The differences in the mean CSF lactate concentration, and in the lactate/pyruvate ratio were not statistically significant ($p > 0\ 05$)

Table II gives all the directly measured tissue parameters as well as the calculated lactate/pyruvate ratios There were no significant differences in the CO_2, ATP, ADP AMP or phosphocreatine contents between the two alkalotic groups ($p > 0\ 05$) However the superimposed metabolic alkalosis gave rise to significant increases in the lactate ($p < 0\ 005$) and in the pyruvate ($p < 0\ 05$) contents and in the lactate/pyruvate ratios ($p < 0\ 05$)

The mean values for the derived intracellular parameters are given in Table III When the group with pure respiratory alkalosis was compared to the group with combined respiratory and nonrespiratory alkalosis the latter was found to have

TABLE III Calculated intracellular bicarbonate lactate and pyruvate concentrations (mEq/kg of i.c. water), lactate/pyruvate and NADH/NAD+ ratios, and pH values for rat brain tissue in respiratory and in combined metabolic and respiratory alkalosis The tissue CO_2 tensions ($PtCO_2$) were derived from the $paCO_2$ values (see Methods) Means ± S E The normocapnic control values are from the series published by Nilsson and Siesjo (1970)

Exper group	$PtCO_2$	HCO_3^-	pH_i	La	Py	La/py	NADH/NAD+
Normocapnia n = 7	45 6 ±1 5	13 24 ±0 43	7 09 ±0 02	1 77 ±0 08	0 106 ±0 007	16 9 ±0 1	2 32 10⁻³ ±0 17
Respiratory alkalosis n = 6	22 8 ±0 3	7 09 ±0 20	7 12 ±0 01	5 04 ±0 19	0 241 ±0 005	20 9 ±0 6	3 03 10^{-3} ±0 09
Metabolic and respiratory alkalosis n = 5	23 3 ±0 5	7 33 ±0 29	7 12 ±0 01	6 75 ±0 53	0 280 ±0 011	24 0 ±1 3	3 52 10^{-3} ±0.21

significantly higher lactate ($p<0.02$) and pyruvate ($p<0.05$) concentrations but the differences in the lactate/pyruvate ratio and in the NADH/NAD ratio were not statistically different at the 5 % level

Discussion

The present results seem to be relevant to two questions The first of these has been discussed in the introduction and concerns the possibility that extreme hyperventilation by a critical lowering of the cerebral blood flow leads to tissue hypoxia in the brain The second question is related to the effect of a superimposed metabolic alkalosis when the arterial CO_2 tension has been lowered below 20 mm Hg As remarked in the introduction this effect would be expected to decrease the oxygen available to the brain even further due to its effect on the oxygen affinity of the red blood cells (Bohr shift)

The present results fully confirm the findings previously reported for cats under phenobarbital anesthesia (Granholm and Siesjo 1969) and for rats under nitrous oxide anesthesia (Kjällquist *et al* 1969) in showing that extreme hyperventilation leads to highly significant increases in the extra and intracellular lactate and pyruvate concentrations as well as in the corresponding lactate and pyruvate ratios Since the increase in the lactate/pyruvate ratio occurs with an alkaline shift in pH^i there is an increase in the calculated cytoplasmatic NADH/NAD ratio *cf* Granholm *et al* 1969) We may assume that such an increase reflects an oxygen-dependent change in the oxido-reduction state of the cell (see *e g* Krebs and Veech 1970 Bücher 1970), *i.e* impaired oxygenation However as long as the hyperventilated state is not acompanied by any significant decreases in the labile phosphate compounds of the tissue hypoxia cannot with certainty be said to be present (*cf* Williamson *et al* 1971) At any rate if extreme hyperventilation leads to cerebral hypoxia

(see Introduction) the present results would suggest that the hypoxia is of moderate degree In fact, the most striking results of studies of the cerebral energy metabolism in hypocapnia may be the absence of marked metabolic changes in spite of the marked reduction in both the pCO and the cerebral blood flow

The results obtained indicate that the combination of respiratory and metabolic alkalosis leads to a moderate further increase in the intracellular lactate (and pyruvate) concentrations but to no significant changes in the CSF lactate concentration or in the CSF pH It is tempting to conclude that the increase in the tissue lactate concentration is caused by the effect of the nonrespiratory alkalosis on the delivery of oxygen to the tissue but the nonsignificant increases in the lactate/pyruvate and NADH/NAD+ ratios, and the absence of any decrease in the phosphocreatine content suggest that any impairment of the oxygen supply must be of very moderate degree

The results offer no explanation to the increase in cerebral blood flow which was reported by Wollman *et al* (1968) under similar experimental conditions unless it is assumed that the moderate aggravation of the intracellular lactacidosis can be made responsible for the increase in flow Whatever is the mechanism responsible for this increase it seems reasonable to assume that it serves to protect the tissue from the harmful effect of a decreased oxygen supply

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Comparative Effects of Angiotensin and Noradrenaline on Resistance, Capacitance, and Precapillary Sphincter Vessels in Cat Skeletal Muscle

By

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Abstract

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The effects of intra arterially infused angiotensin II and *l* noradrenaline on consecutive vascular sections were studied in a sympathectomized skeletal muscle region in the cat under conditions of constant perfusion pressure Noradrenaline was found to be an effective constrictor of pre capillary resistance vessels and capacitance vessels but a poor constrictor of precapillary sphincters whereas angiotensin was an effective constrictor of precapillary resistance vessels and precapillary sphincters , but a poor constrictor of the main capacitance vessels Nor adrenaline caused a net fluid absorption and angiotensin a net filtration across the capillaries which indicated that noradrenaline was a poor but angiotensin an effective constrictor of post capillary resistance vessels

Angiotensin is considered the most potent vasoconstrictor agent formed in the organism Under normal circumstances, however the role of angiotensin in the control of the circulatory system may be limited to the renal vasculature since the endogenously produced plasma concentrations of the substance in the general circulation are quite small (Catt *et al* 1969) It is possible on the other hand that the general systemic vascular effects become significant in pathological states such as hypertension that *are characterized by considerably raised plasma angiotensin concentrations* (Catt *et al* 1969) Pronounced vascular adjustments are elicited by angiotensin when administered in large amounts for instance when it is used as a pressor agent in the clinic

Much work has been done to elucidate the mode of action of angiotensin on the peripheral circulation For instance attempts have been made to analyse the effects on the various consecutive sections of the peripheral vascular bed (see Mellander and Johansson 1968) and such studies have revealed that angiotensin is a more effective constrictor of resistance than of capacitance vessels This was first shown

by Folkow *et al* 1961 in a region consisting of skeletal muscle and skin and has later been confirmed repeatedly in various tissues (*e g* Haddy *et al* 1962, Texter *et al* 1964, Boatman and Brody 1964) Although it thus is agreed that angiotensin mainly affects precapillary resistance vessels there is some evidence that in addition, it has some constrictor effect on small veins (Kettel *et al* 1964, Emerson *et al* 1965, Vacek 1966, Abboud 1968)

The effects of angiotensin on capillary exchange functions as mediated by adjustments of the pre to postcapillary resistance ratio and of precapillary sphincter activity have not been analysed in detail previously In the present investigation an attempt was made to study these effects of angiotensin in a skeletal muscle region in the cat Such studies would elucidate to what extent this agent might change the fluid distribution between the intra and extravascular compartments and affect the size of the functional capillary surface area available for exchange In addition analysis were performed of the concomitantly evoked effects in the resistance and capacitance vessels For comparison a reference substance, noradrenaline was used the vascular effects of which are known in some detail from previous investigations (see Mellander and Johansson 1968)

Methods

Observations were made on 15 cats ranging in weight from 2 2 to 5 4 kg The cats were anesthetized i.v with a mixture of chloralose (50 mg/kg) and urethane (100 mg/kg) and a few of these animals received pentobarbital sodium (10—20 mg/kg) as well The results were similar in all animals regardless of the anesthetics used

The study was performed on an acutely denervated region consisting of the muscles in the lower leg Briefly the skin was separated from the leg muscles so that it had no blood supply but could be used to cover the calf muscles during the experiment The paw was removed at the ankle joint and the femur was drilled open and plugged Thus all vascular connections to the lower leg muscles except the popliteal artery and vein were removed The sciatic nerve was cut After heparinization the popliteal vein was cannulated and venous outflow of blood from the muscle region recorded continuously with a drop recorder The arterial inflow was diverted from the proximal part of the femoral artery via a T tube catheter to the popliteal artery This was done to permit close arterial infusions of angiotensin and noradrenaline A small mixing chamber was inserted in the arterial shunt circuit to ensure even distribution of the vasoactive agents to the region Arterial inflow pressure was monitored from the femoral artery of the opposite limb and venous outflow pressure from the cannulated popliteal vein The lower leg was placed in a water filled temperature controlled plethysmograph to permit continuous recording of changes in tissue volume

With this technique it is possible to follow the reactions within the resistance and capacitance vessels the precapillary sphincters as well as net transcapillary fluid movements (cf Mellander and Johansson 1968) The resistance function was obtained from pressure/flow recordings Changes of precapillary sphincter activity were deduced from observed alterations of the capillary filtration coefficient (CFC) assuming that the agents did not change capillary permeability CFC was determined by raising venous outflow pressure a known amount and recording the rate of net filtration caused by the associated increase of capillary hydrostatic pressure In all calculations of CFC the pre to postcapillary resistance ratio was taken to be 4/1 (cf Pappenheimer and Soto-Rivera 1948 Cobbold *et al* 1963) implying that 80 per cent of the venous pressure rise was transmitted to the capillary level The responses of the capacitance vessels were determined from the initial and rapid changes in tissue volume occurring during the administration of the substances These reflect changes in regional blood volume The rate of net transcapillary fluid movement caused by the agents was assessed from slower continuous changes in tissue volume observed in a steady state phase of drug action In some experiments the capacitance responses were recorded concomitantly to the volumetric method by external monitoring the regional changes in radioactivity of the red cells labelled with ^{51}Cr (for details see Åblad and Mellander 1963 Kjellmer 1965)

Angiotensin II amide (Hypertensin Ciba) was infused in doses ranging from 0.25 to 2.5 μg/kg tissue × min and l noradrenaline base in doses of 0.5 to 4.5 μg/kg tissue × min. Both substances were dissolved in saline and infused intra-arterially at a constant rate of 0.18 ml/min.

In 4 experiments the adrenals were ligated in order to eliminate possible release of catecholamines.

Results

In the control period before the administration of the vasoactive agents venous outflow pressure was adjusted so as to establish a normal transmural pressure in the capillaries and thus an 'isovolumetric state' of the studied skeletal muscle region. Under these circumstances, when venous transmural pressure is also in the normal range, the regional blood volume in denervated skeletal muscle of the cat is about 3 ml/100 g tissue (*e.g.* Mellander 1960) and this was taken as the control value for the blood volume in the capacitance vessels. CFC was determined repeatedly at intervals and the resistance function was recorded continuously in the control period. The mean control values (±SEM) for regional resistance was 13.8±1.0 peripheral resistance units (mm Hg/(ml/min×100 g tissue)), and for CFC 0.010±0.001 ml/min×100 g×mm Hg. Angiotensin (AT) was infused only once in each experiment to avoid tachyphylaxis, whereas noradrenaline (NA) was given in repeated infusions both before and after AT and in doses eliciting resistance responses of similar magnitudes in the individual experiment as evoked by AT.

The constrictor responses of the resistance vessels to both substances started soon after the commencement of the infusions and reached steady levels within 2 to 4 min. Coordinated with the resistance increase, tissue volume showed an initial rapid decline reflecting the constrictor response of the capacitance vessels; steady state resistance and capacitance responses were thus established within about the same period of time. The resistance effect was usually maintained at a steady level throughout the period of infusion and so was the capacitance response as evidenced by the shape of the volume curve and in particular by the results obtained with the ^{51}Cr isotope technique. During the phase of steady state resistance and capacitance responses tissue volume showed a slow but continuous change reflecting net transcapillary fluid movement. The slope of this volume change was recorded for about 3 min after which CFC was determined. Upon cessation of the infusions of AT and NA vascular tone returned to the control level, usually within about 5 min. The above described pattern of vascular response showing well maintained steady state effects was elicited in most experiments.

The results to be presented below refer to the type of experiment mentioned in the foregoing in which the various vascular effects could be evaluated readily in quantitative terms. In a few cases the resistance response to AT was however not maintained at a steady level during prolonged infusion but showed a gradual decline. Such experiments had to be excluded since they did not permit a clearcut distinction between capacitance effects and net transcapillary fluid movements.

Arterial blood pressure remained constant during the intra-arterial infusions of the agents and no systemic effects were revealed. When comparable doses of the

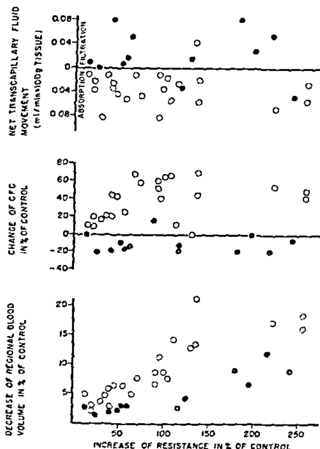


Fig 1 Collected data to show the effects on the consecutive sections of the vascular bed in the sympathectomized skeletal muscle during close arterial infusions of angiotensin (closed circles) and noradrenaline (open circles) The effects on capacitance vessels (change of regional blood volume), on precapillary sphincters (change of CFC), and on net transcapillary fluid movement are plotted against the concomitant effects on the resistance vessels

substances were given no major difference was observed between the results obtained in cats with intact and with ligated adrenals Since, furthermore, the region was sympathectomized the evoked effects can be considered the result of a direct action of the agents on vascular smooth muscle

The results are shown in Fig 1 and 2 which summarize all data obtained In Fig 1 the change in the resistance function is plotted along the abscissa and the other vascular responses along the ordinates This permits to evaluate at a given level of resistance vessel constriction the simultaneously observed effects within the other consecutive vascular sections The effects of the substances on the resistance and capacitance vessels as well as on CFC are expressed in per cent of the control values before the start of the infusions The rates of the induced net transcapillary fluid movements are expressed in absolute figures Fig 2 shows the mean values $\pm$ SEM for the various vascular responses

It can be seen (Fig 1) that AT (closed circles) and NA (open circles) always caused a constriction of the resistance vessels To obtain equally great resistance responses in the individual experiments NA had to be given in larger amounts than

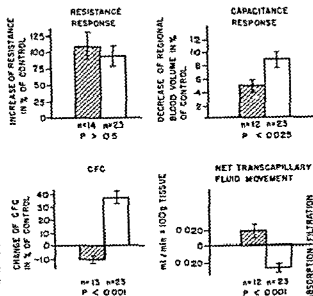


Fig. 2. Mean values ($\pm$SEM) for all observations of the effects on the resistance vessels, capacitance vessels, capillary filtration coefficient (CFC) and on net transcapillary fluid movement of i.a. infusions of angiotensin (shaded columns) and noradrenaline (white columns). The number of observations (n) and significance levels (P) are indicated below the columns.

AT. The mean values of the doses used in all experiments were 2.7 μg/kg$\times$min for NA and 1.0 μ/kg$\times$min for AT. Thus, when given in equiweight doses, AT was a more effective constrictor of resistance vessels than NA. The data in Fig. 1 show that by adjustments of the doses the elicited resistance effects varied within roughly the same range for the two substances. On the average, resistance increased by 108 $\pm$ 20 per cent for AT and by 94 $\pm$ 15 per cent for NA (Fig. 2). There is no significant difference between these effects ($P > 0.5$). Although the resistance effects of these agents thus can be considered quite comparable, this does not seem to be true for the other vascular responses. Thus, the constrictor response of the capacitance vessels appeared to be less pronounced for AT than NA. This difference may be most clearly seen in the upper range of resistance response. On the average AT caused a decrease of regional blood volume by 4.9 $\pm$ 1.0 per cent and NA by 8.6 $\pm$ 1.2 per cent. This difference is statistically significant ($P < 0.025$). With only few exceptions, AT elicited a moderate decrease of CFC (mean value—10 $\pm$ 3 per cent) whereas NA caused an increase of CFC (mean value +39 $\pm$ 5 per cent). This difference is significant ($P > 0.001$). Finally, AT almost invariably led to a net transcapillary filtration of fluid from the intravascular to the extravascular compartment (mean 0.019 $\pm$ 0.012 ml/min$\times$100 g tissue), and NA to a net fluid absorption (mean 0.035 $\pm$ 0.006 ml/min$\times$100 g), ($P > 0.001$).

The extent to which mean hydrostatic capillary pressure was changed by these agents can be calculated roughly by dividing the figure for the observed rate of net transcapillary fluid movement by the concomitantly determined value for CFC. On the average, AT caused a rise in mean hydrostatic capillary pressure by 2.1 $\pm$ 1.3 mm Hg and NA a decrease by 2.6 $\pm$ 0.5 mm Hg.

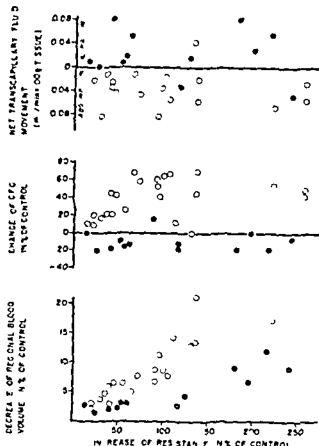


Fig. 1. Collected data to show the effects on the consecutive sections of the vascular bed in the sympathectomized skeletal muscle during close arterial infusions of angiotensin (closed circles) and noradrenaline (open circles). The effects on capacitance vessels (change of regional blood volume), on precapillary sphincters (change of CFC) and on net transcapillary fluid movement are plotted against the concomitant effects on the resistance vessels.

substances were given no major difference was observed between the results obtained in cats with intact and with ligated adrenals. Since furthermore the region was sympathectomized the evoked effects can be considered the result of a direct action of the agents on vascular smooth muscle.

The results are shown in Fig. 1 and 2 which summarize all data obtained. In Fig. 1 the change in the resistance function is plotted along the abscissa and the other vascular responses along the ordinates. This permits to evaluate at a given level of resistance vessel constriction the simultaneously observed effects within the other consecutive vascular sections. The effects of the substances on the resistance and capacitance vessels as well as on CFC are expressed in per cent of the control values before the start of the infusions. The rates of the induced net transcapillary fluid movements are expressed in absolute figures. Fig. 2 shows the mean values ±SEM for the various vascular responses.

It can be seen (Fig. 1) that AT (closed circles) and NA (open circles) always caused a constriction of the resistance vessels. To obtain equally great resistance responses in the individual experiments NA had to be given in larger amounts than

on the resistance vessels This conclusion seem to be corroborated by the finding of the present study performed on a pure skeletal muscle region The data obtained permit a more strict quantitative evalution of these differences than was possible previously and over a wider range of elicited vascular effects (Fig 1 and 2) The analysis further suggested that some 40 per cent of the total capacitance response to AT could be ascribed to a passive emptying of these vessels secondary to constriction on the arterial side and hence decreased transmural pressure in the veins Therefore, the true active contraction of the smooth muscle in the capacitance vessels (mainly veins) was quite, although not entirely negligible

The present data can provide some information about the effects of AT and NA on the ratio of pre- to postcapillary resistance and on the precapillary sphincters These effects of AT have not been studied in detail before When arterial inflow pressure and venous outflow pressure are kept constant, a relatively more pronounced increase of prethan postcapillary resistance leads to a fall in mean hydrostatic capillary pressure and hence to an absorption of extravascular fluid to the circulatory system Such an effect was obtained with NA in agreement with previous results (Mellander 1960 Mellander 1966) A similar response would *a priori* have been expected with AT in view of the marked constriction of the resistance vessels (situated mainly on the precapillary side) concomitant to the comparatively small constriction of the capacitance vessels (situated mainly on the postcapillary side) The results showed however that on the contrary AT usually caused a net filtration of fluid into the tissue indicating a decrease of the ratio of pre to postcapillary resistance Earlier studies have suggested that at least in some tissues the small veins can constrict in response to AT (Kettel *et al* 1964 Emerson *et al* 1965 Vacek 1966, and Abboud 1968) and these findings may help to explain the phenomenon described above It is possible that these small veins constitute the main postcapillary resistance vessels and that therefore moderate constrictions here can influence the pre to postcapillary resistance ratio significantly It should be realized that in absolute terms postcapillary resistance is much smaller than precapillary resistance the ratio being about 4/1 in resting muscle A moderate increase of postcapillary resistance may thus balance or overbalance a great increase of precapillary resistance to the extent that this ratio is maintained or even decreased It appears from the present direct observations of net transcapillary fluid movement that AT by constricting both precapillary and postcapillary resistance vessels can elicit such an effect It may be concluded that the postcapillary resistance and capacitance functions are not necessarily confined to the same types of vessel and that vasoactive substances can effect these functions in dissimilar manners the effect of AT on the capacitance function seems to be very small This view is consonant with the observation that another vasoactive agent, dihydroergotamine does not affect the ratio of pre to postcapillary resistance despite the fact that it elicits a very strong constriction of the capacitance vessels concomitantly to a moderate dilatation of the resistance vessels in skeletal muscle (Mellander and Nordenfelt 1970) In a recent study Abdel Sayed *et al* (1970) reported a somewhat

greater increase of small vein pressure in skeletal muscle in the dog in response to NA than to AT under circumstances when the total regional resistance increase was about equally great for the two substances. Theoretically, such data might suggest that AT would cause a more rapid fluid absorption than NA, an opinion contrasting to the results of the present study in which net transcapillary fluid movement was recorded directly. Although species differences may well exist, it appears that observations of small vein pressure *per se* do not always permit conclusions to be drawn about the effects on net transcapillary fluid movement. This because an increased postcapillary resistance may reside in smaller venous vessels than those giving rise to increased small vein pressure upon constriction. If, as assumed in the present study, AT preferentially constricts the smallest postcapillary vessels but not the large ones, a fall in small vein pressure can be expected when measured with the technique used by Abdel Sayed *et al.* A remote possibility that, in our experiments, AT might have caused some sludging and hence increased postcapillary resistance seemed refuted by the fact that all vascular effects, including the fluid filtration, were eliminated quickly upon cessation of the AT infusion.

The smooth muscle activity of the precapillary sphincters determine the number of patent capillaries and hence the size of the functional capillary surface area available for filtration and diffusion exchange. Changes in the capillary filtration coefficient have commonly been used as a measure of changes in precapillary sphincter activity to physiological and pharmacological stimuli (Mellander and Johansson 1968), assuming that the stimuli do not affect the other determinant of CFC, i.e. capillary permeability. So far there seems to be no experimental evidence that invalidates this assumption with regard to the action of NA and AT.

CFC was found to be increased considerably above the control level during NA infusion (Fig. 1 and 2) indicating a relaxation of precapillary sphincters and hence increased functional capillary surface area. This effect probably is not a direct effect of NA but an indirect one since previous studies have shown that the precapillary sphincters are under dominant influence of the main local control mechanisms, i.e. chemical metabolic factors and myogenic mechanisms (Mellander and Johansson 1968). A passive reduction of blood flow is known to lead to a pronounced relaxation of precapillary sphincters due to accumulation of dilator metabolites and to abolition of myogenic tone consequent to the reduced transmural pressure (Cobbold *et al.* 1963). This effect was also demonstrated in the present study. NA may elicit a very transient constrictor effect in precapillary sphincters but, since the substance also reduces flow and vascular transmural pressure, this effect is evidently not maintained in the steady state phase of NA action due to the associated interference with the normal local control mechanisms (*cf.* Mellander 1966). In contrast to this effect observed during NA administration, AT was shown to be able to decrease CFC below the control level in the steady state. This strongly suggests that the direct constrictor effect of AT on the precapillary sphincters is so marked as to effectively oppose the counteracting influences of the local control mechanisms. The latter influences must be considered about equally great with

NA and AT, since the flow reductions were comparable for both substances In fact, AT appears to be the only substance known so far that can elicit a maintained constriction of the precapillary sphincters in the face of a significant flow reduction (*cf* Mellander 1970)

It can be argued however, that owing to the different effects of NA and AT on the pre- to postcapillary resistance ratio, CFC might have been overestimated for NA, but underestimated for AT CFC was determined in response to a standardized increment of venous outflow pressure, assuming in all calculations that 80 per cent of this increase was transmitted to the capillary level (pre- to postcapillary resistance ratio of 4/1) Since NA was shown to increase, and AT to decrease, this ratio, the induced capillary pressure rise should have been somewhat greater for NA and somewhat lower for AT than assumed The consequent error in the CFC determination can be calculated, however, to be no greater than about 4 per cent Even if this is taken into consideration, the difference between the two substances with regard to their effects on CFC is still highly significant

It may be concluded that NA is an effective constrictor of precapillary resistance vessels and capacitance vessels, but a poor constrictor of precapillary sphincters and postcapillary resistance vessels AT, on the other hand, is an effective constrictor of precapillary and postcapillary resistance vessels and of precapillary sphincters, but a poor constrictor of the main capacitance vessels

This pattern of vascular response in skeletal muscle may, or may not be present in other tissues The finding that AT is a relatively more effective constrictor of postcapillary than precapillary resistance vessels in muscle leading to net filtration may have a correlate in the kidney AT is reported to increase glomerular filtration rate by causing a more pronounced constriction of the efferent than afferent arteriole of the glomerulus (Schmid 1962 Gross *et al* 1965, Ziegler and Janzik 1968)

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Intravascular Concentrations of Calcium and Magnesium Ions and Edema Formation in Isolated Lungs

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Abstract

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The perfusate concentration of Ca^{++} has been assumed to be important for the permeability of the exchange vessels to proteins low [Ca^{++}] is said to result in edema development The possible role of perfusate [Mg^{++}] in this connection has previously not been considered In the present work the correlation between the perfusate concentrations of Ca^{++} and Mg^{++} and edema development has been analyzed

In a perfused rabbit lung preparation edema developed at physiological intravascular pressures when the perfusate (plasma) [Ca^{++}] was reduced to about $7 \cdot 10^{-4}$ mM and the [Mg^{++}] to about $1 \cdot 10^{-4}$ mM by EDTA additions Edema development was not seen at intravascular concentrations of Ca^{++} and Mg^{++} of about $4 \cdot 10^{-4}$ and $4 \cdot 10^{-3}$ mM respectively Such concentrations were achieved by addition of EGTA to the perfusate Thus either the [Mg^{++}] or the [Ca^{++}] plus [Mg^{++}] in the perfusate must be the important parameter for the edema development The changes in the capillary permeability induced by EDTA additions were largely reversible by addition of Ca^{++} This effect of Ca^{++} had a latency period of less than one min

The capillary wall behaves as a selective diffusion barrier between the blood and the interstitium, but the mechanisms of this barrier function are not fully understood Mainly on the basis of experiments performed by Chambers and Zweifach (1940) it has been generally accepted that the concentration of calcium ions is an important factor for the maintenance of normal capillary permeability These authors observed that edema developed in frog mesenterium when its vessels were perfused with a calcium free Ringer solution which also contained sodium oxalate A few other reports have strengthened the impression that calcium ions are of importance for capillary permeability In a rat hind limb preparation Wilbrandt Luscher and Asper 1956) thus found a reduction in the effective colloid osmotic pressure when ethylenediaminetetraacetic acid (EDTA) was added to the perfusate Spector 1957 found that injection of sodium citrate into the skin of rats increased the local capillary permeability He presumed this to be due to removal of calcium ions from the tissue

The permeability of other cellular membranes also appears to be affected by calcium Hays Singer and Malamed 1965) thus found the toad bladder wall to have

an increased permeability to electrolytes and to inulin when it was kept in a fluid free from added calcium Curran, Zadunaisky and Gill (1961) demonstrated increased conductance of the toad skin indicating increased permeability to electrolytes when EDTA was added to a calcium free medium surrounding the organ

In these two tissues toad bladder wall and frog skin it has been found that maintenance of normal permeability cannot be achieved when the calcium ions are replaced by magnesium ions (Lipson Dodelson and Hays 1965, Curran Zadunaisky and Gill 1961) On the other hand magnesium ions have been shown to be more effective than calcium ions in restoring the permeability of the rat intestinal wall after an EDTA exposure (Tidball 1964) The possible role of magnesium ions for the permeability of capillaries has not been discussed in the literature The reported changes in capillary permeability claimed to be induced by low [Ca] could however well be related to low [Mg] as well

The mechanisms by which low [Ca] and or low [Mg]) increases the permeability of cellular membranes are not known A common hypothesis is however that the effect of low [Ca^{++}] is to weaken cell adhesion (Chambers and Zweifach 1940 Hays Singer and Malamed 1965) Goldner 1967) however believes that the increased permeability of the rat intestinal wall after exposure to EDTA is due to changes in the membrane of the epithelial cell rather than to changes at the cell junctions

In order to obtain more information about the role of Ca and or Mg for the permeability of capillaries I have investigated the quantitative relationship between the intravascular concentrations of Ca and Mg on the one hand and edema formation on the other Furthermore the reversibility of such edema development has been studied To this end an experimental technique was used where edema formation in perfused rabbit lungs could be continuously and quantitatively followed Horse plasma turned out to be the most suitable perfusate in most of the experiments and its [Ca] and [Mg] was varied by addition of the chelators EDTA and ethyleneglycoldiaminetetraacetic acid (EGTA) Preliminary reports on some of these experiments have been given elsewhere Nicolaysen 1969 Nicolaysen 1970

Methods

The lung p eṭa a on were taken fr all n rabbi ts f eitl er sex e gh ng 2 3 5 kg The abb s vere anestl et zed w tl pent barl tone Ne nbutal g Al b tt 30 50 r g kg Hep ar 750 The j edure The hest la n was acc l n the c d aro nd ent

n l pro e F g l s a schen at c dra ing of t e exp r

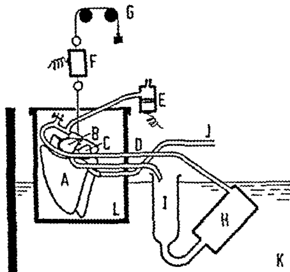


Fig 1 Schematic drawing of the perfusion and recording arrangement for the isolated lung preparation
A Lung preparation B The nonbeating heart C Left auricle with perfusion outflow cannula D Perfusion inflow cannula (into pulmonary artery) E Pressure transducer for recording of pulmonary arterial pressure F Force transducer for recording of changes in preparation weight G Counter balance system for the force transducer H Perfusion pump I Perfusate reservoir J Cannula for ventilation (into trachea) K Thermostatically controlled waterbath L Organ chamber

Ventilation Immediately after start of perfusion the lungs were inflated and positive pressure ventilation started using a Starling 'Ideal' pump (C F Palmer (London), Ltd) (22 strokes min) The inspiratory peak pressure and minimum expiratory pressure were set at 10 and 1 5—2 cm of water, respectively In most experiments 5 per cent CO_2 in air was used for ventilation During some experiments the CO_2 content of the gas was gradually reduced in order to avoid pH values below 7 2 of the perfusates

Weighing The preparation was placed inside a plastic casing and suspended by the string around

[illegible]

Perfusates Three different types were used

a) *Heparinized horse plasma* obtained by centrifugation of heparinized (3000 I U 100 ml of pure powdered heparin Novo horse blood for 10 min at about 1000 g The plasma was divided in [illegible] experiment a plasma portion was thaw

[illegible] es of anesthetized (10—30 mg kg [illegible] ts 2000 I L of heparin per 100 ml

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sorptio n photometer respectively. Some measurements of concentration of ionized calcium were done with an Orion calcium-specific electrode.

In two experiments the magnesium content in the lung tissue was measured by activation analysis at Institutt for Atomenergi, Kjeller, Norway.

Experimental design

Direct measurement of the concentration of ionized calcium in biological fluids seems at present to be possible only at concentrations exceeding about 0.1 mM. Preliminary experiments indicated that one had to aim at concentrations much below this value. This introduced problems both as regards evaluation of ion concentration and also for the selection of perfusion fluids. Artificial perfusates in which the concentrations of the actual ions could be set at various selected levels could not be used for long-term perfusion of rabbit lungs as spontaneous edema formation will start about 30–40 min after start of perfusion (Nicolaysen, unpublished). As a result of this, either whole blood or plasma perfusates had to be used in most experiments. The concentration of ionized calcium and magnesium in these perfusates could be reduced by addition of chelating agents. The chelating agents which form stable complexes with calcium also form complexes with magnesium although less stable ones (Ringbom 1963). The binding of both metals imposed difficulties for a differentiation between effects of reduced concentrations of calcium ions and of magnesium ions. The use in the present study of two different chelating agents allowed, however, some degree of differentiation.

In a plasma perfusate it was also relatively easy to estimate, on the basis of calculations, the actual concentration of calcium ions [Ca] and magnesium ions [Mg]. Such calculations were rather uncertain for whole blood, due to the presence of red cells with their content of magnesium. One could not predict the extent to which erythrocyte magnesium would be available for chelation in the blood plasma. For these reasons plasma was chosen as perfusate in most of the present experiments.

In a series of more than 10 experiments it has been shown (Nicolaysen, in preparation) that the preparation can be perfused with horse plasma at physiological pressures for at least 4 h without spontaneous edema formation taking place. All tests in the present experiments were applied within a 4 h period. The use of horse plasma made it possible to perform series of experiments with different batches from the same original plasma portion.

Weight increase of the preparation was used as a measure of edema formation. Lunde (19) found that when isolated perfused rabbit lungs had gained 4 g in weight during stable pressure conditions, then edema of the preparation was undoubtedly present. The weight of total extravascular tissue is about 8 g in a non-edematous preparation of this type (Lunde 196). This degree of weight increase was taken to indicate that definite edema of the preparation had been reached.

Results

Development of edema in plasma perfused lungs on addition of EDTA. The stability constants of both calcium and magnesium EDTA complexes are high. EDTA addition to a plasma perfusate can therefore reduce both the concentration of calcium ions and of magnesium ions. In a series of experiments the effects as regards edema development upon addition of EDTA to the plasma peerfusate were evaluated. In the experiment depicted in Fig. 2 portions of a 67 mM solution of Na_2EDTA in saline (EDTA) were repeatedly added to the perfusate reservoir. Up to the point of the 6th addition only small and inconsistent changes in both directions of the weight of the preparation and of the P A p occurred. About 15 min after the 6th addit[illegible] however, an accelerating increase in weight was evident. At the sa[illegible] the P[illegible] wed a slight decrea[illegible] Some 25 min after the 6th addition d[illegible] the preparation was present.

An evaluation of the exact time cour[illegible] attempted when a sufficiently high EDT[illegible] ed. Early in the experiment of Fig. 3 [illegible] giving a

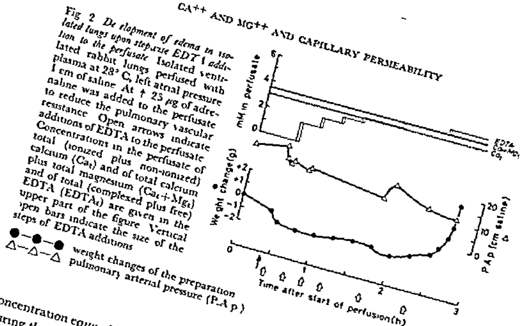


Fig 2 *Development of edema in isolated lungs upon stepwise EDTA addition to the perfusate* Isolated ventilated rabbit lungs perfused with plasma at 28° C, left atrial pressure 1 cm of saline At ↑ 25 μg of adrenaline was added to the perfusate to reduce the pulmonary vascular resistance Open arrows indicate additions of EDTA to the perfusate Concentrations in the perfusate of total (ionized plus non-ionized) calcium (Ca_t) and of total calcium plus total magnesium ($Ca_t + Mg_t$) and of total (complexed plus free) EDTA ($EDTA_t$) are given in the upper part of the figure Vertical open bars indicate the size of the steps of EDTA additions
●—●—● weight changes of the preparation
△—△—△ pulmonary arterial pressure (P.A p.)

concentration equivalent to 95 per cent of ([Ca_t] plus [Mg_t]) Edema did not develop during the next two hours of perfusion Then another dose of EDTA was added, and now a weight increase became apparent within 10 min The weight increase accelerated and definite edema was present about 30 min after the last addition of EDTA This pattern of development was always seen upon addition of sufficiently large doses of EDTA (8 experiments) and irrespective of whether the addition was made in an early or late part of the perfusion In all these experiments the P A p was below 20 cm of saline throughout the perfusion When applying these facts to the experiment of Fig 2, it appears that the edema formation was initiated when the concentration of total EDTA (complexed plus free, [$EDTA_t$]) reached 4 mM (about 115 per cent of ([Ca_t] plus [Mg_t])) and not as a result of the previous and lower concentrations of EDTA

It was then established (6 experiments) that when the EDTA concentration in the perfusate was increased stepwise as in the experiment of Fig 2, edema always devel-

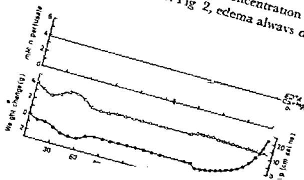


Fig 3 *Time course of the edema development produced by EDTA addition to the perfusate* Preparation as in Fig 2 Symbols and abbreviations as in Fig 2 Note that the time scale has been expanded as from 160 min after start of perfusion

oped when the concentration of EDTA was 120 per cent of ([Ca$_t$] plus [Mg$_t$]) in the plasma

In order to test the effects of subthreshold doses of EDTA two other experiments similar to that of Fig 3 were performed An EDTA concentration of 95 % (1 experiment) or 85 % (1 experiment) of the sum of total calcium and total magnesium concentrations did not cause any sustained weight increase within a two hour period This indicates that there is no long term tendency towards edema formation at such EDTA concentrations

The calculated (see Appendix) plasma levels of [Ca^{++}] and [Mg^{++}] at EDTA concentrations of 95 per cent and 120 per cent of ([Ca$_t$] plus [Mg$_t$]), respectively, are given in Table I At the latter EDTA concentration the concentration of Ca^{++} in the plasma would be as low as $6.8 \cdot 10^{-4}$ mM and the concentration of Mg^{++} $1.1 \cdot 10^{-2}$ mM

Thus both [Ca^{++}] and [Mg^{++}] were considerably reduced at the EDTA levels which caused edema formation in these preparations The effect of the EDTA addition on the preparation could then be due to the reduced concentration of Mg^{++} as well as to the reduced concentration of Ca

The effect of isolated reduction in [Ca^{++}] An attempt was made to separate the effects of low concentrations of Ca and of Mg by using the chelating agent ethyleneglycoldiaminetetraacetic acid (EGTA) instead of EDTA For the calcium and magnesium complexes with EGTA the conditional constants (stability constants at prevailing conditions) at pH 7.4 are $10^{7.8}$ and $10^{1.7}$, respectively For the EDTA complexes these values are $10^{7.8}$ and $10^{5.8}$ respectively Ringbom 1963 Thus the preference for calcium binding over magnesium binding is much stronger for EGTA than for EDTA At the same time the conditional constants for calcium at the pH used are about the same for the two chelating agents

In 3 experiments EGTA (67 mM solution in saline, pH 8) was added stepwise to the plasma perfusate reservoir One of the experiments are shown in Fig 4 In all three experiments a concentration of EGTA corresponding to 150 per cent of [Ca$_t$] plus [Mg$_t$]) was reached *without* edema development taking place After subsequent addition of (in 2 of the experiments) EDTA in an amount (in mmoles equal to

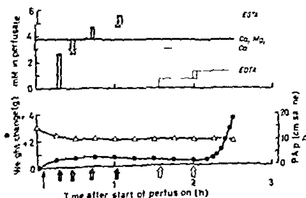


Fig 4 *Effect of stepwise addition of EGTA to the plasma perfusate of isolated lungs* Preparation as in Fig 2 At t was added 7.5 mg papaverine sulfate to the perfusate At cross hatched arrows additions of EGTA and at open arrows additions of EDTA to the perfusate Cross-hatched and open bars indicate the size of the steps of EGTA and of EDTA additions respectively EGTA indicates the concentration of total EGTA (complexed plus free) in the perfusate Other symbols and abbreviations as in Fig 2

about 1/4 of the EGTA previously added, then edema developed (Fig 4) At the 150 per cent level of EGTA in the plasma the Ca^{++} and Mg^{++} concentrations were estimated by calculation to be $4\ 2\ 10^{-6}$ mM and $4\ 4\ 10^{-1}$ mM, respectively Thus at a Ca^{++} of $4\ 2\ 10^{-6}$ mM edema did develop in all the EDTA experiments but not in any of the EGTA experiments There are two possible explanations for this either the [Mg^{++}] alone or the sum [Ca^{++}] plus [Mg^{++}] is the important parameter for the edema development This sum was much higher in the EGTA experiments than in the EDTA experiments, $4\ 4\ 10^{-1}$ mM in the former as against $1\ 1\ 10^{-3}$ mM in the latter

Effects of isolated reductions in Mg^{++} concentration In order to test the effects of very low concentrations of Mg^{++} at a normal level of [Ca^{++}] an artificial perfusate was used The experiments could then only last for 30—40 min (see Experimental design) In three experiments a Krebs-Ringer Dextran solution with no magnesium added was used as perfusate In order to avoid contamination of the perfusate with blood from the pulmonary vessels the first 100 ml of perfusate leaving the lungs after start of perfusion were discarded In addition a perfusate exchange was carried out twice during the first 15 min of perfusion In the subsequent 20 min observation period no weight increase was seen in any of the experiments In a similar experiment with a Krebs-Ringer Dextran perfusate containing magnesium, during which EDTA was added to reach a concentration equal to 120 per cent of the ([Ca_t] plus [Mg_t]), an accelerating weight increase was apparent within 10 min In the former three experiments, [Mg_t] in the perfusate was found to be within the range of $1\ 10^{-2}$—$1\ 5\ 10^{-2}$ mM during the observation period (after the second change of perfusate) The concentration of Mg^{++} reached was thus considerably lower than those obtained in the EGTA experiments, but not as low as the ones in the EDTA experiments where edema was released It is therefore not possible from these experiments to ascertain whether the reduction in [Mg^{++}] alone or in the ([Mg^{++}] plus [Ca^{++}]) is the important parameter for the edema development upon EDTA additions to the perfusate

Reversibility of the effects caused by very low [Ca^{++}] and [Mg^{++}] In a separate series of experiments the reversibility of the effects of edema producing concentrations of EDTA was tested by adding a 0 1 M $CaCl_2$ solution to the perfusate reservoir The molar amount of calcium added represented 1/3—1/8 of the molar amount of EDTA previously added to produce the edema formation In these experiments papaverine sulfate (5—7 5 mg/200 ml) had to be added to the perfusate from the outset This prevented a severe constriction of resistance vessels which were otherwise induced by increasing the Ca and Mg^{+} concentrations from the extremely low values achieved by EDTA addition $CaCl_2$ was added in 3 experiments where the weight of the preparation was rising but before definite edema (as earlier defined) had been established The $CaCl_2$ addition was thus presumably made after an outward flux of fluid from the capillaries had started In all these experiments the $CaCl_2$ addition markedly affected the further weight development (Fig 5) A change in the weight curve was apparent within 1—2 min The weight did either become stable (Fig 5) or the rate of the weight increase was considerably reduced Only small or no changes

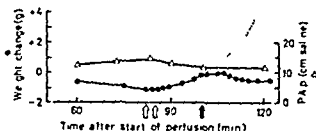


Fig 5 Effect on isolated lungs of adding CaCl₂ to the plasma perfusate during edema formation produced by previous EDTA addition Preparation as in Fig 2 Papaverine sulfate (7 5 mg) had been added 10 min after start of perfusion At filled arrow addition of $CaCl_2$ to the perfusate indicate the curve of expected weight change if $CaCl_2$ addition had been omitted Other symbols and abbreviations as in Fig 2

in vascular resistance was induced by the addition of $CaCl_2$ In two experiments where $CaCl_2$ was added subsequent to an edema provoking EDTA dose, but before any weight increase had started, no weight increase appeared Addition of $CaCl_2$ had little effect where it was postponed until definite edema of the preparation had occurred

The marked effects of $CaCl_2$-additions in these experiments unfortunately give no information as to the relative role of calcium and magnesium ions in maintaining normal capillary permeability Addition of $CaCl_2$ in these experiments increases the Mg^{++} as well as the Ca^{++} concentrations some of the added calcium will release magnesium from its EDTA-complex

Experiments with homologous rabbit whole blood as perfusate Three perfusions were done with whole blood as a perfusate and at 38 C An EDTA solution was added stepwise to the reservoir Fig 6 shows the results of one of these experiments After addition of the 4th EDTA dose an accelerating weight increase began Three min later the weight had increased by 1 4 g and at that point $CaCl_2$ was added to the reservoir This addition resulted in a transient weight decrease and a transient increase in P A p Shortly thereafter a slow increase in weight developed — probably reflecting some continued

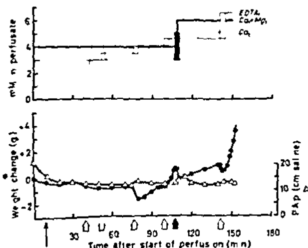


Fig 6 *Edema formation in perfused lungs on EDTA addition to the whole blood perfusate and the effect of $CaCl_2$ on this edema development* Perfusion performed with whole blood as a perfusate and at 38 C Otherwise same type of preparation as in Fig 2 At * 7 5 mg of papaverine sulfate was added to the perfusate EDTA additions at open arrows At filled arrow a dose of $CaCl_2$ and 25 µg of adrenaline was added to the perfusate In the upper part of the figure are given the concentrations in the *blood plasma* of Ca_t and of $Ca_t - Mg_t$ and also the presumed concentration of EDTA Symbols and abbreviations as in Fig 2

edema formation in the lower parts of the lungs The rate of the weight increase, however, was much slower than that just before the addition of $CaCl_2$ A new dose of EDTA (equimolar to the $CaCl_2$ dose previously added) again produced an accelerating weight increase, and in 15 min massive edema of the preparation had developed As can be seen from the figure, edema development was produced when the EDTA concentration in the blood plasma had reached about 115—120 per cent of plasma ($[Ca_t]$ plus $[Mg_t]$) (provided that the EDTA did not diffuse into red cells within this time) The time from the EDTA addition to the start of weight increase was much shorter in this type of experiments than in the plasma experiments carried out at 28° C Identical results were obtained in the two other experiments with whole blood as perfusates In these 3 experiments adrenaline (25 μg) had to be added together with the $CaCl_2$ solution Addition of $CaCl_2$ alone released an intense vasoconstriction even where papaverine had previously been added to the perfusate

Magnesium content of the lung tissue proper The values presented for $[Ca^{++}]$ and $[Mg^{++}]$ in the perfusates at different concentrations of EDTA and EGTA are based on the assumption that the magnesium of the lung tissue proper was not available for chelation This might, however, not be the case The only report on lung Mg content which is available (Rams, Eichelberger and Moulder 1969) indicate a concentration of about 4 mmoles/kg of magnesium in lung preparations containing some intravascular fluid The magnesium content of the lung tissue *proper* was evaluated in two pairs of lungs in the present investigation The calcium content in the lung tissue was assumed to be negligible in relation to the amount of calcium in the 200 ml plasma perfusate used and was therefore not measured The lungs were ventilated and perfused with plasma for 15 min, then deflated and the perfusate allowed to drain from the pulmonary artery and the pulmonary veins The right and left lung from each pair were then weighed, cut into small pieces and their total magnesium content determined Lunde (1967) showed that in deflated rabbit lungs of the same size the amount of perfusate remaining in vessels after perfusate drainage was about 37 per cent of the weight of the deflated lungs The $[Mg_t]$ of the plasma perfusate was determined Thus the Mg content of the lung tissue proper could also be evaluated The concentration of magnesium in the lung tissue proper was in this way found to be 6 5 mmoles/kg wet tissue

Discussion

The method of continuous weighing of a preparation makes it possible to follow incipient edema development provided that concomitant alterations in intravascular volume can be evaluated or neglected An increased intravascular volume could be a result of an increased transmural vascular pressure or a decreased tension in the vascular wall Intravascular pressures as judged from pulmonary arterial and left atrial pressures did, however, remain nearly constant during the critical periods in most of the present experiments It is conceivable that changes in vascular tension have taken place in the present tests It is improbable, however, that marked altera-

tions in vascular elasticy should have occurred in this preparation with its low vascular tone. Only edema development can then explain the acceleration and the extent of the weight increase observed. The edema development in isolated lungs is also known to follow an accelerating course (Levine, Mellins and Fishman 1965, Lunde 1967).

Edema development at unhampered lymphatic drainage can be caused by changes in capillary pressure or by changes in capillary permeability. In the present experiments the lymph vessels from the lungs were divided, but not ligated so that lymphatic drainage into the perfusate reservoir could occur freely. Since the pulmonary inflow- and outflow-pressure remained nearly constant and at physiological levels, and since the pressure gradients over the vascular bed were small, only slight changes in capillary pressure can have taken place. From other studies (Lunde and Waaler 1969) it is also known that increases in the pulmonary capillary pressures of a few mm of Hg from a physiological level results only in *transient* outward flux of fluid from the capillaries. It is reasonable then to conclude that the weight increments in the present experiments were predominantly due to net outward fluxes of fluid from the capillaries as a result of changes in capillary permeability. Only changes in capillary permeability to large molecules (albumin, Dextran) will cause disturbance of the transvascular fluid balance. It can then be concluded that the capillary permeability to proteins (or Dextran) had increased in the present experiments when edema developed.

Concentrations of EDTA above a given limit thus decisively increased the vascular permeability to large molecules since spontaneous edema development is not seen during a 1 h perfusion of the preparation (see Experimental design). Wilbrandt, *Lüscher and Asper (1950) also reported from the application of an isogravimetric* technique, that EDTA could increase the capillary permeability to proteins. Direct toxic effects of the CaEDTA and MgEDTA complexes could not be the cause of the edema development seen, since in the experiments where the effects of low [Ca^{++}] and [Mg^{++}] were reversed high concentrations of these complexes did not produce edema. A direct toxic effect of EDTA separate from its complexation of metals seems improbable. The effect of EDTA of increasing the permeability is therefore most certainly due to its complexation of metals in the perfusates, with the consequent reduction in the concentrations of the ionized forms of these metals. In plasma there are traces of metals other than calcium, magnesium and sodium that form complexes with EDTA and EGTA. The sodium chelation with EDTA could be shown by calculations to be negligible (see Appendix). That the chelation of trace-metals should be the cause of the edema seen in the present experiments cannot be excluded. Such a role of the trace-metals has, however, never been discussed. The most likely explanation for the effects of adding EDTA or EGTA must be that the substances act by reducing the concentration of calcium and magnesium ions.

The calculations of the [Ca^{++}] and the [Mg^{++}] have been based on several assumptions. An important one was that the chelating agents remained chemically active in the perfusates *during perfusion. Popovici et al. (1950) found that the complex of*

calcium with EDTA forms stoichiometrically in rabbit serum at physiological pH Some measurements in plasma of [Ca^{++}] by a calcium selective electrode (Orion) at EGTA and EDTA concentrations up to 90 per cent of the Ca_t concentration, showed that at least in this range and under the prevailing conditions the reductions in [Ca^{++}] corresponded to the expected degree of chelation These observations seem to justify the assumption that the chelators remained active in plasma

The conditional constant (= stability constant at the prevailing conditions) (Ringbom 1963) of EGTA for sodium chelation was not known According to Ringbom (1969 personal communication) it is, however, reasonable to assume that EGTA has the same or a smaller conditional constant for sodium chelation than has EDTA The sodium chelation with EGTA has therefore been disregarded Only if the conditional constant for the NaEGTA chelate were much higher than that for the NaEDTA-chelate would this affect some of the conclusions reached

The experiments presented indicate that either the [Mg^{++}] or ([Mg^{++}] plus [Ca^{++}]) in the perfusate is a critical parameter for maintenance of low capillary permeability to proteins The EGTA experiments show that rather extreme reduction in [Ca^{++}] at nearly maintained [Mg^{++}] do not affect this property of the capillaries If the intravascular ([Ca^{++}] plus [Mg^{++}]) is the critical parameter, then Ca^{++} and Mg^{++} must probably be freely interchangeable at the sites determining permeability in the capillary wall Differentiation between the parameters ([Ca^{++}] plus [Mg^{++}]) and [Mg^{++}] required perfusates in which the [Mg^{++}] could be reduced to 1 1 10^{-3} mM or less at maintained normal concentrations of Ca^{++} Clearly one would want to devise experiments which would permit conclusions as to this point, no such experiments are, however, at present available

Edema formation was produced when the concentration of Mg^{++} and of (Mg^{++} plus Ca^{++}) was reduced to about 1 10^{-3} mM but not when it was at levels of about 4 5 10^{-1} mM Because of the uncertainties inherent in the system, no attempts were made to define the concentration limits more exactly In two experiments, however, EDTA in amounts about equal to plasma [Mg_t] could be added in addition to previously added EGTA equaling 150 per cent of ([Ca_t] plus [Mg_t]) without edema development (Fig 4) These experiments indicate that the critical concentrations of Mg^{++} or of (Mg^{++} plus Ca^{++}) must thus be well below 4 5 10^{-1} mM

Any protein binding of calcium and magnesium in the plasma perfusate would result in lower values for [Ca^{++}] and [Mg^{++}] at the different concentrations of the chelating agents than estimated by the calculations performed in this work

In rabbit lungs the Mg content was found to be about 6 5 mmoles/kg lung tissue Thus a pair of rabbit lungs contain about 0 05 mmole Mg whereas there is about 0 1 mmole in the 200 ml of plasma perfusate used Because of this quite large tissue Mg content, a second set of calculations were made, taking the lung tissue Mg into account and treating it as if it were dissolved in plasma The calculations show that there would be relatively small changes in calculated [Ca^{++}] and [Mg^{++}] at concentrations of chelating agents exceeding ([Ca_t] plus [Mg_t]) (Table I) It also seems reasonable to presume that only a small amount of the lung tissue Mg will be available for chela-

tion with EDTA and EGTA. Therefore the values obtained for $[Ca^{++}]$ and $[Mg^{++}]$ when the lung tissue Mg is disregarded are probably the more relevant ones.

The present experiments give no direct information as to the mechanisms of the increased permeability at the low Ca^{++} and Mg^{++} concentrations. Some experimental studies (Danielli 1940, Gimbrone et al. 1969) present evidence for an important role of thrombocytes in endothelial maintenance. A change in thrombocyte function at low $[Ca^{++}]$ and $[Mg^{++}]$ seems to be an improbable explanation for the present results. Thus Lunde (1967) found that the presence or absence of circulating thrombocytes was not critical with respect to edema formation in isolated perfused rabbit lungs. In the present experiments the number of thrombocytes in the plasma perfusates was not controlled but must have been extremely low as a result of the centrifugation, freezing and filtration procedure followed. The current concept is that the intercellular clefts are the pathways for most molecules moving across the walls of capillaries with continuous endothelium (as in lungs). The effect of reduced $[Ca^{++}]$ and/or $[Mg^{++}]$ could possibly result from a widening of these clefts by weakening of cell adhesion. Zeidman (1947) found that magnesium as well as calcium are essential for maintenance of normal cell adhesiveness in human buccal mucosa. The experiments of Forte and Nauss (1963) and of Sedar and Forte (1964) indicate that changes in the cell junctions of bullfrog gastric mucosa induced by low $[Ca^{++}]$ could be reversed by calcium but not by magnesium addition. There thus seems to be species (and/or organ) differences as to the effects of magnesium on cell adhesion. The results of the present experiments therefore do not give specific information on this point. Changes in pinocytotic activity or in other transcellular transport mechanisms could also be the cause(s) of the edema-giving changes in protein permeability. It is hoped that experiments on isolated lungs and involving electron microscopic examination of the capillaries and their surroundings, might reveal more about the effects of low $[Ca^{++}]$ and $[Mg^{++}]$. Such experiments are in progress.

The effects of low ($[Ca^{++}]$ plus $[Mg^{++}]$) or $[Mg^{++}]$ were to a large extent reversible in the experiments here reported. The experiments of Wilbrandt, Luscher and Asper

TABLE I. Calculated concentrations of Ca^{++} and Mg^{++} in the plasma perfusates at different plasma concentrations of chelating agents. In part I only plasma calcium and magnesium are considered available for chelation, in part II lung tissue magnesium is also included.

	Concentration of chelating agent	3.33mM EDTA	4.2mM EDTA	5.25mM EGTA
	Concentration of calcium (ionized plus non ionized)	3mM	3mM	3mM
	Concentration of magnesium (ionized plus non ionized)	5·10[illegible] mM	5·10[illegible] mM	5·10[illegible] mM
I	Concentration of Ca	1.5·10[illegible] mM	6.8·10[illegible] mM	4.2·10[illegible] mM
I	Concentration of Mg	1.7·10[illegible] mM	1.1·10[illegible] mM	4.4·10[illegible] mM
II	Concentration of Ca	3.0·10[illegible] mM	1.1·10[illegible] mM	4.2·10[illegible] mM
II	Concentration of Mg^{++}	4.2·10[illegible] mM	2.6·10[illegible] mM	5·10[illegible] mM

(1956) also indicated that EDTA effects on capillaries could be reversed. Similar information has also been found for other cellular membranes (Curran, Zadunaisky and Gill 1961, Tidball 1964, Lipson, Dodelson and Hays 1965). One point deserves attention. In the plasma experiments at 28° C there was a latency period of about 10 min after the critical reduction in concentration of calcium and magnesium ions before any definite weight effect could be seen. In the whole blood experiments performed at 38° C this latency period was reduced to a few min (Fig. 6). The effect of adding $CaCl_2$, however, was evident in both situations within one min from the addition. Thus it seems that the "opened up channels" must be blocked at the very first contact with intravascularly increased concentration of calcium or magnesium ions. The same time pattern of responses to changes in Ca^{++} concentration was found by Curran, Zadunaisky and Gill (1961) in experiments on the frog skin.

Permeability increase resulting in edema formation may be considered as an extreme change of the vascular membrane. The possibility exists that more moderate reductions in concentrations of Ca^{++}, Mg^{++} or in both could increase capillary permeability, although not to such an extent that edema develops. Experiments designed to analyze this problem are now being carried out (Nicolaysen in preparation).

Appendix

Calculations

The $[Ca^{++}]$ and $[Mg^{++}]$ in the plasma perfusates at different concentrations in the plasma of the chelating agents EDTA and EGTA, were calculated in the following way and on the basis of the following assumptions.

1) Total concentrations of calcium $[Ca_t]$, (ionized plus non ionized) and of magnesium $[Mg_t]$ in the plasmas were measured.

2) All plasma calcium and magensium not bound to EDTA or EGTA were assumed to be ionized.

3) It was assumed that the concentrations in plasma of metals other than calcium, magnesium and sodium which could be chelated by EDTA or EGTA were negligible.

4) Sodium chelation with EDTA can be assumed, on the basis of calculations, to be negligible at the actual concentrations. The conditional constant for the NaEDTA chelate at pH 7.4 is $10^{1.8}$ (Ringbom 1963) so $\frac{[NaEDTA]}{[Na]\,[EDTA]} = 10^{-1.8}$. At the concentrations of EDTA used, the Na^+ concentration in the plasma (0.14 M) will be nearly unchanged whether EDTA chelates plasma sodium or not. It follows $\frac{[NaEDTA]}{[EDTA]} = 10^{-1.8} \cdot 0.14 \approx 1 \cdot 10^{-3}$. Thus only a negligible fraction of the added EDTA can be present in the form of Na chelate in the plasmas.

5) In the first set of calculations (Table 1) it was assumed that the magnesium of the lung tissue proper would not be available for chelation. In the second set the lung tissue magnesium was assumed to be available for chelation.

6) The chelating agents were assumed to have the same conditional constants for the calcium, magnesium and sodium chelates in plasma as in a aquous solution of the same pH.

A. At an EDTA concentration in the plasma perfusate equaling 95 per cent of ($[Ca_t]$ plus $[Mg_t]$) the following equations were used:

1) $$\frac{[CaEDTA]}{[Ca^{++}]\,[EDTA]} = K_{CaE}$$

2) $$\frac{[MgEDTA]}{\quad}$$

etc.

In Equation 5) it was assumed that all added EDTA were present in the form of complexes
K_{CaL} at pH 7.4 = $10^{7.9}$, K_{MgL} at pH 7.4 = $10^{6.4}$ (Ringbom 1963). This system of equations was solved for [Ca^{++}] by first dividing equation 1 by equation 2, and then eliminating [CaEDTA] and [MgEDTA] by the use of equations 3 and 4. The [Mg^{++}] was then at last eliminated by the use of equation 5. When the [Ca^{++}] had been calculated, the [Mg^{++}] was found by equation 5. When the magnesium of the lung tissue was taken into account this amount of magnesium was treated as if it were dissolved in the plasma.

B. At $EDTA_t$ = 120 per cent of ([Ca_t] plus [Mg_t]) the following equations applied:

a) $\frac{[CaEDTA]}{[Ca^{++}][EDTA]} = K_{CaL}$

b) [CaEDTA] = [Ca_t] *Approximately all calcium were in the form of chelates.*

c) [EDTA] = [$EDTA_t$] − [Ca_t] − [Mg_t]. In this equation the approximation was made that all calcium and magnesium was in the form of chelate.

...] was calculated. The [Mg^{++}] was calculated as follows:

... −[Ca_t] same approximation as in equation b.

m) $\frac{[MgEDTA]}{[Mg^{++}][EDTA]}$ $10^{6.4}$

From these equations [Mg^{++}] was calculated. The [Mg^{++}] and [Ca^{++}] were also calculated at this EDTA level taking the lung tissue Mg into consideration. Again this was done by the appropriate ...

... as [Mg_t]. The ... 120 per cent ...

B

I want to thank Mr. R. Sothe for skilful technical assistance. This work has been supported by grants to the Institute of Physiology from *Hjelpestikkene*, The Nansen Foundation and The Norwegian Research Council for Science and the Humanities. The powdered heparin was generously supplied by Novo Industri A/S, Oslo and Copenhagen.

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Changes in the Action Potential and Contraction of Isolated Frog Muscle after Repetitive Stimulation

By

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Abstract

HANSSON J and A PERSSON *Changes in the action potential and contraction of isolated frog muscle after repetitive stimulation* Acta physiol scand 1971 81 340—348

The effect of repetitive stimulation of twitch fibres was studied in isolated whole muscle from Rana pipiens Intracellular electrodes were used for recording membrane potentials muscle tension was recorded isometrically and the decline of the active state of the contractile elements was determined by the quick release method Repetitive stimulation caused an increase in the duration of the action potential and in the amplitude of the early negative after potential To judge from experiments on fibres with disrupted transverse tubules the increase in the duration of the action potential is due to changes in the surface membrane while the increase in the amplitude of the after potential is related to the transverse tubules The changes in the action potential and the after potential were independent of whether or not the muscle contracted and thus cannot be secondary to the contraction The shape of the action potential and the after potential returned to normal within 5 min after stimulation at 10/sec for 60 sec while after 30 min there was still an increase in the peak twitch tension and a slower than normal decay of the active state in response to test shocks. This indicates that the post stimulatory potentiation is due to a prolonged active state which in turn is due mainly to causes other than changes in the action potential and after potential

Repetitive stimulation of muscle fibres causes an increase in the duration of the action potential an increase in the amplitude of the early negative after-potential and as well a decrease in the resting potential and in the peak amplitude of the action potential (Persson 1963 Luttgau 1965 Colomo and Rocchi 1965) Luttgau found that in single frog fibres the decline in amplitude and the prolongation of the action potential were much less if muscle contraction was prevented and concluded that these changes were effects of the contraction process Observations made in the course of a previous investigation (Persson 1963) have led us to question the validity of this statement Luttgau also found that the action currents recorded extracellularly, change before any decrease in muscle tension is observed This is contrary to the findings of Merton (1954 in man In Luttgau s experiments the extracellularly recorded action currents recovered after a brief tetanus at about the same rate as the

post tetanic enhancement of the peak twitch tension decreased Colomo and Rocchi (1965), on the other hand, found no clear relationship between the size of the peak twitch tension and the amplitude of the action potential recorded intracellularly

The present investigation was undertaken to study the changes in the shape of the action potential and peak twitch tension produced by repetitive stimulation at a frequency likely to occur under physiological conditions and to determine whether any clear relationship exists between the two parameters The action potential (recorded intracellularly), the isometric mechanical tension and the time course of the decay of the active state were examined Experiments were made on whole muscles to avoid the risk of membrane damage in single fibre preparations during repetitive stimulation

Methods

Preparation The experiments were performed on the sartorius and extensor digitorum longus IV (EDL IV) muscles of English frogs (Rana pipiens) The animals were kept in a cold storage room at 4° C

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Recording Capillary micro electrodes filled with 3 M KCl were used for intracellular recording of the membrane potential Only electrodes with a resistance of 5—10 MΩ were used The micro-electrode was connected through an Ag AgCl electrode to the input cathode follower of a differential DC amplifier (grid current 10^{-11} A, input capacity 5pF) The reference electrode in the external solution was also Ag AgCl

In order to avoid damage to the fibre the micro electrode was generally withdrawn from the fibre during the period of repetitive stimulation In some experiments the fibre was impaled only after the stimulation period to ensure that previous impalement would not make any difference to the

u o which the muscle tendon was tied

The potentials were displayed on a double-beam cathode ray oscilloscope and recorded on film

Excitation of the muscle fibres was obtained in three ways

1) A stimulating current was passed through a Ringer filled glass pipette with a tip pore of about 100 μ m diameter If the tip of the electrode was placed near the surface of the muscle excitation was limited to a few fibres and disturbing movements were minimized

2) In experiments on the EDL IV muscle in which the effects of repetitive stimulation on the action potential and the twitch tension were studied simultaneously the whole muscle was stimulated directly through Ag AgCl electrodes The stimulus strength was set well above that which gave maximal twitch amplitude to a single shock Neuromuscular transmission was blocked by adding tubocurarine chloride (5 μ g/ml) to the Ringer fluid

3) The muscle fibres were stimulated via the nerve which was dissected free for a few cm

In some experiments the falling phase of the active state was determined by the quick release method (Ritchie 1954) under conditions similar to those in 2)

Solutions Ringer's solution had the following composition (mM) NaCl 110 KCl 2 5 $CaCl_2$ 2 0 $NaHCO_3$ 2 5 Na_2HPO_4 1 2 NaH_2PO_4 0 6 glucose 10 Hypertonic solutions were made by adding glycerol (400 mM) or sucrose (240 or 290 mM) to the Ringer fluid In some experiments the muscle was poisoned by adding NaCN (2 or 4 mM) to the Ringer solution The pH of the solutions ranged from 7 2 to 7 4

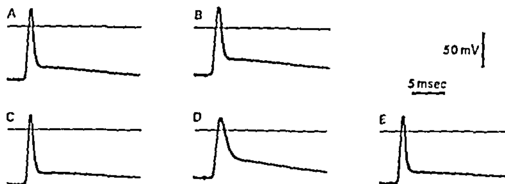


Fig. 1. Action potentials. *A* before, *B* immediately after stimulation at 10/sec for 20 sec, *C* after 5 min rest, *D* immediately after stimulation at 10/sec for 60 sec, *E* after 5 min rest (sartorius muscle). In this and subsequent records level of zero potential indicated by horizontal bar. All action potentials from same fibre.

Results

The effect of repetitive stimulation on the action potential

Fig. 1 illustrates the effect of repetitive stimulation on the shape of the action potential. After 15—20 sec of stimulation at 10/sec there occurred an increase in the duration of the action potential, due mainly to a decrease in the rate of decline of the action potential, and an increase in the peak amplitude of the early negative after-potential (Fig. 1 B). The resting potential and the peak amplitude of the action potential were only slightly decreased immediately after the period of stimulation. The increase in the late negative after potential (Freygang *et al.* 1964), if present, was therefore small and of short duration. The changes were more pronounced after longer stimulation (Fig. 1 D). After one min stimulation at 10/sec the resting potential and the peak amplitude of the action potential were generally decreased; after longer stimulation the action potentials became so deformed that the duration and the amplitude of the negative after potential could not be measured adequately. The changes were always of the same kind in both sartorius and EDL IV, but could vary in degree between different animals and also from fibre to fibre in a preparation from the same animal.

The changes in the amplitude of the early negative after potential (measured 4 msec after the beginning of the action potential) following stimulation at 10/sec for 60 sec are illustrated in Fig. 2 A. Fig. 2 B shows the corresponding changes in the duration of the action potential, measured as the interval between the points of intersection of a line at the level of the membrane potential preceding the action potential and the tangents through the inflexion points.

The changes in the action potential were equally pronounced in directly stimulated fibres and in fibres stimulated via the nerve. Whether the muscle fibres had been impaled or not before the stimulation made no apparent difference. This excludes the possibility that the changes in the action potential were due to muscle fibre damage.

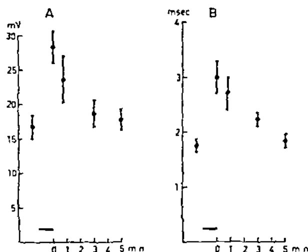


Fig 2 The effect of stimulation at 10/sec for 60 sec on the amplitude of the after potential (A) and the duration of the action potential (B) Stimulation indicated by horizontal bar Mean of 104 action potentials Length of vertical bars = standard deviation Experimental points represent observations from different fibres (Sartorius)

The effect of different stimulation frequencies was also investigated It was found that the changes in the action potential increased with the number of repetitive stimuli at any one stimulus frequency Stimulation at 10/sec was found to be more effective than 1/sec and 50/sec Furthermore, to evoke the aforementioned changes in the action potential required about twice as long a period of stimulation at 10° C than at room temperature (Fig 3)

Changes in the action potential during repetitive stimulation in non contracting fibres

In experiments on single isolated fibres Luttgau (1965) found that when the fibre is prevented from contracting repetitive stimulation produces no or very small changes in the action potential regardless of whether muscle contraction was blocked mechanically for example with hypertonic solutions or chemically with metabolic inhibitors

In our experiments immersion of the muscle in hypertonic sucrose Ringer's solution greatly reduced or abolished the twitch within one minute The muscles (sartorius and in some experiments also EDL IV) were stimulated directly The dura

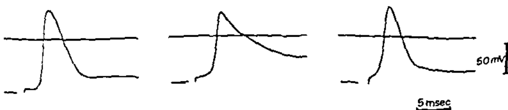


Fig 3 Action potentials at 10° C before immediately after stimulation at 10 sec for 2 min and after 10 min rest. (Sartorius)

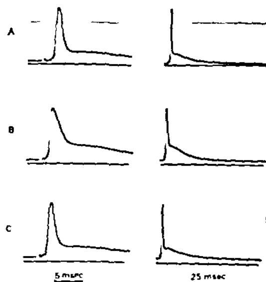


Fig. 4. Contraction blocked in Ringer solution made hypertonic with 290 mM sucrose (Sartorius). Action potential and tension (lower sweep in each record). *A* before, *B* immediately after stimulation at 10/sec for 60 sec, *C* after 8 min rest.

tion of the action potential and the amplitude of the negative after potential were about 15 % greater in sucrose Ringer than in ordinary Ringer (sartorius). As shown in Fig. 4 changes in the action potential and the after potential similar to those described earlier were obtained after repetitive stimulation. In 16 fibres (sartorius) that after stimulation for one hour at 10/sec gave an action potential the duration and after potential of which could be measured adequately, the duration increased from 2.02 ± 0.28 msec (mean ± SD) to 4.18 ± 1.00 msec after stimulation and the amplitude of the negative after potential increased from 19.5 ± 1.2 mV to 30.1 ± 1.4 mV. For a muscle in ordinary Ringer's solution the corresponding changes were an increase in ordinary Ringer's solution the corresponding changes were an increase in duration from 1.75 ± 0.21 msec to 2.98 ± 0.64 msec and an increase in the amplitude of the after potential from 16.8 ± 3.4 mV to 28.3 ± 4.9 mV. The peak amplitude of the action potential decreased by about 10 % in ordinary Ringer's solution and 20 % in sucrose Ringer.

Fibres whose contractility was exhausted following immersion in NaCN Ringer were also examined (sartorius and EDL IV). Even when no muscle contraction was detectable it was still possible to record action potentials but they were considerably increased in duration and distorted. No further changes in the action potential were observed during repetitive stimulation.

In other experiments (sartorius) contraction was blocked by disruption of the transverse tubules achieved by first immersing the muscle in glycerol Ringer solution for one hour and then returning it to normal Ringer solution (Howell and Jenden 1967, Gage *et al.* 1967). During this process characteristic changes were seen in the negative after potential. During the phase when a contraction could still be evoked by electrical stimulation there was an increase in the amplitude of the after



Fig 5 Effect of immersion for 1 hour in Ringer solution containing 400 mM glycerol (Sartorius) *A* before, *B*, immediately after return to normal Ringer solution (twitch still present) *C*, somewhat later (no twitch), *D*, immediately after stimulation at 10/sec for 60 sec, *E*, after 5 min rest.

potential (Fig 5 B) When contraction could no longer be evoked the early negative after potential had disappeared (Fig 5 C) Even in these fibres repetitive stimulation at 10/sec for 60 sec caused an increase in the duration of the action potential from 1 87 ± 0 52 msec to 2 96 ± 0 78 msec Just as in the case of normal fibres with intact electromechanical coupling, this increase gradually subsided within about 5 min (Fig 5 D, E) No after potential was seen after the repetitive stimulation The peak amplitude of the action potential declined by about 5 %

After disruption of the transverse tubules the latency of the action potential was about one third of the original, as was to be expected from the diminished membrane capacitance (Gage *et al* 1969)

Effect of repetitive stimulation on the mechanical response

After 15—20 sec stimulation at 10/sec there was either a slight decrease or a slight enhancement in tension with or without fusion of the twitches After 60 sec stimulation at 10/sec the tension had usually decreased to less than half of the initial tension, unless there was a pronounced fusion of the twitches in which case the tension might remain at the level of the initial twitch peak Upon cessation of such stimula

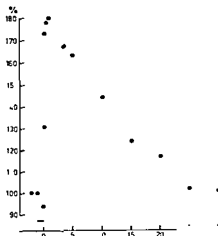


Fig 6 Changes in the amplitude of the twitch on stimulation at 10/sec for 60 sec (EDL IV) Stimulation indicated by bar

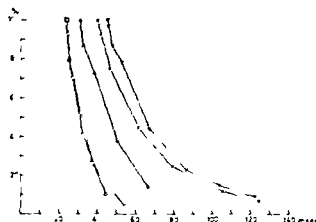


Fig 7 Decay of the active state (Sartorius) □ before ▽ 30 sec after stimulation at 10/sec for 60 sec ⁄ after 5 min rest △ 15 min rest, ○ 45 min rest.

tion there was a marked increase in the amplitude and duration of the twitch (post stimulatory potentiation) which did not decline until after more than 30 min rest (Fig 6 Even stimulation at 10/sec for only 15 sec gave rise to post stimulatory potentiation that persisted for 20 min That the duration of potentiation is of this order of magnitude in twitch fibres was also confirmed by experiments on a few single fibres from the iliofibularis muscle

In order to obtain a measure of how long the contractile elements were activated during each contraction after repetitive stimulation the time course of decay of the active state was investigated in a few experiments As shown in Fig 7 the decay of the active state following stimulation at 10/sec for 60 sec was considerably delayed as compared with that of the control twitch Return to the original curve did not occur before 30 –45 min rest Thus the changes in the twitch tension as well as the decay of the active state following repetitive stimulation persisted considerably longer than the changes in the action potential after corresponding stimulation

Discussion

The occurrence of changes in the shape of the action potential following repetitive stimulation increase in the duration of the spike and increase in the amplitude of the early negative afterpotential—described by earlier workers (Persson 1963 Lüttgau 1965 Colomo and Rocchi 1965) has been confirmed These changes are dependent on the number of stimuli at any one stimulus frequency but are independent of the manner in which the muscle is stimulated directly or via the nerve and are not due to damage to the membrane They appear before any marked decrease is seen in the resting potential or in the peak amplitude of the action potential

In experiments on single fibres Luttgau (1964 1965) found that when contraction is prevented repetitive stimulation produces little or no change in the action potential The present results on the other hand show that repetitive stimulation produces the same type and magnitude of change in the action potential even when mechanical response is suppressed by immersion of the fibre in hypertonic-Ringer

solution In fibres in which excitation contraction coupling is interrupted by disruption of the transverse tubules no after potential is seen (Gage *et al* 1967) However, even in such fibres an increase in the duration of the action potential was observed in the present study Thus the contraction itself does not appear to be the cause of the changes in the action potential that occur during repetitive stimulation

The discrepancy between Luttgau s and the present results is difficult to explain Luttgau worked on another preparation and routinely used considerably higher stimulus frequencies (up to 150/sec) Most of his experiments were performed with extracellular electrodes with this technique it is difficult to adequately measure the duration of the action potential and the amplitude of the after potential which are the parameters primarily changed during repetitive stimulation

The transverse tubules which are responsible for two thirds of the K^+ permeability of the muscle fibre (Eisenberg *et al* 1969) do appear to be of vital importance for the appearance of the early negative after potential the amplitude of which changes in parallel with changes in the size of the transverse tubules that to judge from electron microscopic investigations (Howell and Jenden 1967 Eisenberg and Eisenberg 1968) occur when the muscle is returned to normal Ringer solution after one hour s immersion in glycerol Ringer solution This results in swelling and rupture of the transverse tubules due to lowered osmotic pressure outside the muscle fibre During this process a marked initial increase in the amplitude of the after potential was found Later when the transverse tubules are disrupted no after potential is seen as previously shown by Gage *et al* (1967) Nor was any negative after potential seen in fibres with disrupted tubules after repetitive stimulation

Gage *et al* (1969) suggested that the early negative after potential is the result of accumulation in the transverse tubules of K^+ which leave the fibre during the action potential That this cannot be the case is apparent however from the fact that the negative after potential seen after two stimuli separated by an interval of 4—6 msec is hardly greater than the after potential seen after a single stimulus (Buchthal *et al* 1959 Adrian *et a* 1962 Persson 1963) More probable is a change in the permeability of the membranes of the tubules after the spike of the action potential Repetitive stimulation as in the present experiments may augment this permeability change and/or affect the ion concentrations which influence the membrane potential during the period after the spike

In fibres with disrupted tubules repetitive stimulation caused an increase in the duration of the action potential of about the same magnitude as in normal fibres This increase thus seems to be due to changes in the properties of the surface membrane

The changes in the action potential and the after potential during repetitive stimulation occur before any substantial decrease in mechanical function is observed Ritchie and Wilkie (1955) showed that the active state is prolonged after a brief tetanus The present results show that the decay of the active state is considerably prolonged following repetitive stimulation at 10/sec for 60 sec The duration of the active state probably increases gradually during low frequency stimulation and

would contribute toward maintaining the level of tension during normal protracted muscle contraction

It is possible that the changes in the action potential and the negative after potential are to some extent the cause of the prolongation of the active state It was found that the duration of the action potential was increased by about 75 % following stimulation at 10/sec for 60 sec The increase in the duration of the action potential and the increase in the amplitude of the early negative after potential cause the membrane potential to be above the mechanical threshold for a longer period than normal Moreover it is possible that the mechanical threshold will be lower since as found by Adrian *et al* (1969), the threshold decreases with increasing duration of a depolarizing pulse After repetitive stimulation the active state curve decays at the same rate as the potentiation of the twitch tension indicating that the potentiation is caused by a prolongation of the active state The time course of decay of the action potential and the after-potential is however considerably more rapid (about 5 times) than the decay of the active state curve Thus prolongation of the active state cannot be entirely due to the changes in the action potential and the after potential nor can they have a common cause as suggested by Luttgau (1965) and Close and Hoh (1968)

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Failure of High P_{CO_2} in the Duodenal Bulb to Inhibit Gastric Acid Secretion

By

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Abstract

NILSSON, G and S J RUNE *Failure of high* P_{CO_2} *in the duodenal bulb to inhibit gastric acid secretion* Acta physiol scand 1971 *81* 349—354

Gastric acid secretion was stimulated by intravenous infusion of pentagastrin (ICI 50 123) in dogs with Pavlov pouches and isolated pouches of the duodenal bulb Elevation of the P_{CO_2} to 150 or 700 mm Hg in the bulbar pouches did not reduce the gastric acid output It is suggested that the high CO_2 tension which normally exists in the canine duodenum during periods of stimulated gastric acid secretion is not involved in the activation of the bulbar mechanism inhibiting the gastric acid secretion

Acid perfusion of isolated pouches of the duodenal bulb inhibits gastric acid secretion (Andersson and Uvnas 1961 Anderson Nilsson and Uvnäs 1965 and 1967 Andersson and Nilsson 1969 Nilsson 1969 a b) The mechanism by which this inhibition is induced remains to be characterized Recently it has been shown that the duodenal CO_2 tension is significantly increased during periods of high gastric secretory rates and values around 500 mm Hg are reached after a meal (Rune and Henriksen 1969) Since CO diffuses rapidly through biological membranes (McIver, Redfiels and Benedict 1926) an increase in the luminal P_{CO_2} may induce a change in the P_{CO_2} of the duodenal mucosal cells and thereby contribute to the activation of the inhibitory mechanism This hypothesis is tested in the present work The experiments were performed on dogs with innervated pouches of the stomach and the duodenal bulb Gastric acid secretion was stimulated by continuous i v infusion of pentagastrin (ICI 50 123)

Methods

Surgical procedures

3 mongrel dogs weighing 12—15 kg were provided with mucosal septal pouches of the stomach (Pavlov type and innervated pouches of the duodenal bulb (Andersson and Uvnas 1961) After each surgical procedure and before the start of the experiments the dogs were allowed a period of 3 weeks for recovery

Reagents

Buffer solution I Citrate-HCl buffer solution pH 2 5 (Sörensen)

Buffer solution II The previous buffer solution given a P_{CO_2} of 150 mm Hg by perfusion with pure CO_2 (AGA) for 1 1/2 hr The pH of the solution was unchanged

Experimental procedures

The experiments were started in the morning after the dogs had been fasted for 18—24 hrs Basal acid output from the Pavlov pouches was recorded during 1 hr The gastric secretory responses were collected in 15 min portions and followed for 4—5 hrs The volume was measured and the acidity determined by titration with 0 01 N NaOH using phenolphtalein as indicator

Before the experimental series was started control experiments were performed to determine if acid perfusion of bulbar pouches inhibits gastric acid secretion induced by pentagastrin In these experiments pentagastrin was given as a continuous intravenous infusion in doses producing approximately 30 per cent of the maximal secretory response to pentagastrin After 2 1/2 hrs the bulbar pouches were perfused with 0 1 N HCl for 1 hr at a rate of 60 ml/hr using a pressure of 5—10 cm of water The pH of the effluent perfusate collected during each 15 min period varied between pH 1 1—1 3 After the period of bulbar perfusion the gastric acid secretion was determind for a further 60 min The results from this series of experiment are shown in Table I Perfusion of the duodenal bulb with 0 1 N HCl evoked in all dogs pronounced inhibition of the acid secretion from the gastric pouches inhibition being greatest during the second 1/2 hr of bulbar perfusion Pentagastrin was therefore used as secretory stimulus in all series of the following experiments

In series A gastric secretion was stimulated as in the control experiments The pH in the bulbar pouches was reduced to levels, wich have been shown to be liminal for activation of the bulbar inhibitory mechanism This was attained by perfusion of the bulbar pouches with buffer solution I The perfusion of the bulbar pouches was started simultaneously with the intravenous infusion of pentagastrin After 2 1/2 hrs buffer solution II was substituted for buffer solution I After one hour the bulbar pouches were perfused for another hour with buffer solution I The pH of the effluent perfusates was 2 6 2 8 In series B the secretory responses to graded doses of pentagastrin were determined during perfusion of the duodenal bulb with either buffer solution I and II alternating Each dose of gastrin was given for 1 hr and the dose response curves were constructed from the mean acid output of the last two 15 min periods at each dose level The doses were selected to cover the whole secretory range from very low to maximal doses of pentagastrin

Series C was performed as series A Instead of buffer solutions air was slowly perfused through the bulbar pouch during the control periods whereas pure CO_2 was perfused during the intermediate hour producing an intrabulbar P_{CO_2} of about 700 mm Hg All bulbar perfusions were performed through Foley catheters, which were choosen to prevent leakage through the mucocutaneous fistula.

The pentagastrin was in all experiments given as continuous intravenous infusion by a peristaltic pump (Harvard Apparatus Co Dover, Mass U S A

Statistical evaluation of data

Common methods of analysis of variance were used in the statistical calculation of results (Snedecor 1956

Results

Control experiments

Table I shows that perfusing the duodenal bulb with 0 1 N HCl significantly reduces the gastric acid secretion stimulated with pentagastrin

Series A

No change in the gastric acid secretion was observed when the P_{CO_2} of the buffer solution perfusing the duodenal bulb was increased from 0 to 150 mm Hg The results from these experiments are shown in Table II

TABLE I Effect of 0 1 N HCl in duodenal bulb on secretory responses to low doses of pentagastrin selected to produce secretory responses corresponding approximately 30 per cent of the maximal response to pentagastrin

			Control hour		Half hour periods following control hour			
Dog	Number of expts	Dose of pentagastrin µg/hr	Range of secretory levels meq/15 min	Relative S E of mean of 4 15 min periods %	Mean and range of secretory rate as a percentage of secretory level during the control hour 1	2	3	4
A	3	40	0 24—0 37	11	**44** (33—56)	**12** (8—15)	18 (12—21)	76 (63—94
B	3	40	0 17—0 31	9	**31** (25—34)	**10** (4—17)	21 (14—27	87 75—103
C	3	40	0 19—0 39	8	**46** (39—54)	**20** (14—24)	34 (20—41)	71 (57—94
Mean					40	14	24	78

Perfusion of the duodenal bulb with 0 1 N HCl was performed during half hour periods 1 and 2 and indicated by bold face figures

TABLE II Effect of bulbar perfusion with buffer solution pH 2 5 and P_{CO_2} 150 mm Hg on secretory responses to low doses of pentagastrin selected to produce secretory responses corresponding approximately 30 per cent of the maximal response to pentagastrin

			Control hour		Half hour periods following control hour			
Dog	Number of expts	Dose of pentagastrin µg hr	Range of secretory levels meq/15 min	Relative S E of mean of 4 15-min periods %	Mean and range of secretory rate as a percentage of secretory level during the control hour 1	2	3	4
A	4	40	0 12—0 24	10	**112** (86—136)	**99** (59—165)	99 (73—136)	112 (55—207)
B	3	40	0 12—0 24	18	**115** (106—132)	**132** (83—182)	136 (122—157)	207 (175—297)
C	4	40	0 39—0 70	9	**110** (81—139)	**114** (80—135)	112 (94—143)	107 (82—130)
Mean					112	115	117	142

Buffer solution (pH 2 5) was perfused through the duodenal bulb in all periods During the half hour periods 1 and 2 (bold face figures) this buffer solution was given a P_{CO_2} of 150 mm of mercury

Series B

The acid responses to graded doses of pentagastrin were not influenced by increasing the P_{CO_2} of the bulbar perfusate from 0 to 150 mm Hg (Fig 1)

Series C

The results from this series of experiments are presented in Table III Elevation of the intrabulbar P_{CO_2} by perfusion with pure CO_2 (P_{CO_2} 700 mm Hg) did not reduce the acid output when compared with the gastric acid responses during the precedi

Fig 1

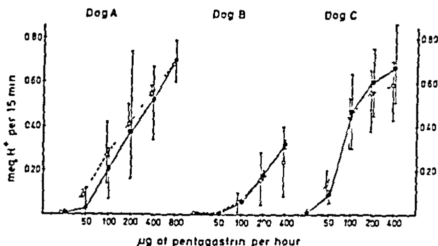


Fig 1 Secretory responses to graded doses of pentagastrin in three Pavlov pouch dogs during perfusion of the duodenal bulb with buffer solution pH 2 5 (closed circles) and the same buffer given a P_{CO_2} of 150 mm Hg (open circles) Each curve represents the mean of 3 expts Vertical bars show the range

TABLE III Effect of bulbar perfusion with atmospheric air and pure CO_2 on secretory responses to low doses of pentagastrin selected to produce secretory responses corresponding approximately 30 per cent of the maximal response to pentagastrin

Dog	Number of expts	Dose of pentagastrin μg/hr	Control hour: Range of secretory levels meq 15 min	Control hour: Relative S D of mean of 4 15 min periods %	Half hour periods following control hour: Mean and range of secretory rate as a percentage of secretory level during the control hour 1	2	3	4
A	3	40	0 28—0 45	15	64 (50—90)	84 (74—98)	85 (32—120	93 (41—133)
B	3	40	0 12—0 31	17	**124** (110—150)	**114** (95—125)	119 (97—139)	105 (93 113)
C	4	40	0 13—0 44	7	**110** (97—127)	**97** (85—106)	89 (73—110)	126 (85—173)
Mean					99	98	98	108

Perfusion of the duodenal bulb with pure CO_2 was performed during half hour periods 1 and 2 and indicated by bold face figures

Discussion

In a series of investigations the mechanism by which acid in the duodenum inhibits gastric acid secretion has been studied These studies show that the area from which inhibition is elicited is localized to the duodenal bulb (Andersson Nilsson and Uvnas 1965 and 1967) *The bulbar mechanism effectively* inhibits acid responses to exog

enous gastrin (Andersson *et al* 1965 and 1967, Andersson and Nilsson 1969) and to stimuli which release endogenous gastrin including test meals (Andersson and Uvnas 1961), insulin hypoglycemia (Nilsson 1969 a) and sham feeding (Nilsson 1969 b The present study shows that acid perfusion of bulbar pouches also inhibits gastric acid responses to the synthetic pentapeptide ICI 50 123, which like gastrin causes stimulation of gastric acid secretion (Barret 1966) The degree of inhibition elicited by bulbar acidification is dependent of pH in the duodenal bulb, inhibition becoming more pronounced the lower the pH (Andersson and Nilsson 1969, Nilsson 1969 b) After a meal the gastric acid secretion is stimulated and the gastric contents become increasingly acid and may when emptied into the duodenal bulb activate the inhibitory mechanism The reasons for testing if the carbondioxide tension in the bulb is of any significance for this inhibition were these

Recent studies have demonstrated that the neutralisation of the gastric contents in the duodenum leads to a very high P_{CO_2} in the proximal part of the duodenum including the bulb (Rune and Henriksen, 1969) After a meal the P_{CO_2} increases to values around 500 mm Hg, and since CO_2 diffuses rapidly through biological membranes (McIver *et al* 1926) an increase in the P_{CO_2} of the duodenal mucosa can be expected Experimental studies have shown that when the pancreatic juice is prevented from reaching the duodenum after a meal a higher gastric acid secretion is found than during normal conditions (Cook, Nahrwold and Grossmann 1967) Those findings may suggest that a high CO_2-tension in the duodenum is necessary for optimal action of the inhibitory mechanism there We tested the significance of this hypothesis under experimental conditions which have been shown to be appropriate for detection of bulbar inhibition of gastric acid secretion (Nilsson 1969 c) Gastric acid secretion was stimulated by doses of pentagastrin which caused the HCl glands to secret slowly pH in the bulbar pouches was reduced to levels which are liminal for activation or partially activates the bulbar mechanism (Andersson and Nilsson 1969 Nilsson 1969 b) Inhibition evoked by elevated CO_2 tension in the bulbar pouches might then be detected as a further reduction of the acid output from the gastric pouches However elevation of the CO_2 tension in the bulbar pouches did not inhibit the gastric acid responses whether the pH in the duodenal bulb was about neutral or reduced to pH 2 5—2 8 by a buffer solution The results from this study therefore suggest that the high P_{CO_2} in the duodenum is not involved in the activation of the bulbar mechanism inhibiting gastric acid secretion

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The Low-Frequency Electrical Impedance of the Isolated Frog Skin

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Abstract

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The electrical impedance of the isolated frog skin has been measured in the frequency range 0 05 Hz—2 kHz Under certain conditions the resistance and capacitance of both outward

The model of frog skin proposed by Koefoed Johnsen and Ussing (1958) pictures the skin as consisting of two cell membranes in series, separated by a layer of cytoplasm, the only structures which significantly impede ion movement are the cell membranes This model is supported by the microelectrode studies of Engbæk and Hoshiko (1957), Ussing and Windhager (1964) and Lindemann and Thorns (1967) in frog skin, and Whittembury (1964) in toad skin, all these workers found discrete jumps in electrical potential when a microelectrode was advanced through the skin epithelium Whittembury (1964) found that the resistance of the inner membrane was ca 30 per cent of that of the outer membrane Lindemann and Thorns (1967) found values varying between 10 and 50 per cent

The variation of the electrical impedance of frog skin with frequency on the other hand is apparently explained by the presence of a single barrier to the movement of ions (Teorell 1949 Brown and Kastella 1965) The capacitance of this barrier has been found to be ca $2 \mu F\ cm^{-2}$ indicative of a cell membrane

In view of the apparent discrepancy between the a c impedance measurements and those made by other methods it was of interest to ascertain whether a more extensive study of the frog skin impedance would also reveal the presence of two cell

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membranes In some of these experiments, the ionic concentration of the inner bathing solution was reduced, in an attempt to make the resistances of the inner and outer membranes more nearly equal

Methods

Experiments were carried out during the months May to July on belly skins of the brown frog (*Rana temporaria*) Skins were mounted in a double-chamber apparatus (Fig 1) with an exposed area of 7.1 cm² In the first part of each experiment either chloride or sulphate Ringer (compositions given in Table I) was used as bathing solution, the solutions were stirred by air bubbling The steady potential difference (p.d.) across the skin was measured *via* Ringer agar salt bridges and calomel half cells using a pH meter (Radiometer model PHM 4b) as voltmeter

The electrodes used for passing current through the skin were discs of either silver or platinum having the same area as the skin At low frequencies bare platinum electrodes had a high polarisation impedance in later experiments therefore the electrodes were [illegible] a layer [illegible] a very [illegible] chloride [illegible] wave [illegible] resistance of 40 kΩ to prevent significant short-circuiting of the skin p.d. (in none of the experiments was the skin short circuited) Two oscillators were needed to cover the frequency range at frequencies above 500 Hz a Hewlett Packard oscillator (model 200 CD) was used and at lower frequencies an instrument constructed in the Ørsted Institute University of Copenhagen The magnitude of the current was measured as the voltage across a standard resistance of 100 Ω in series with the current electrodes using the lower beam DC differential input of a Tektronix 502 oscilloscope

When the skin p.d. became steady after 1 1/2—2 hrs the pH meter leads were detached and the potential electrodes connected to the oscilloscope through 10 x attenuation probes, using the upper beam DC differential input

The oscilloscope was used in the X Y operating condition so that a graph of current against voltage was displayed on the screen For a pure resistance such a graph would be a straight line the frog skin has capacitance as well as resistance so that the graph became an ellipse (Fig 2)

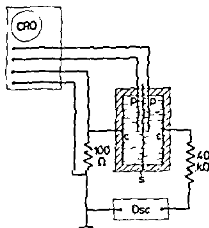


Fig 1

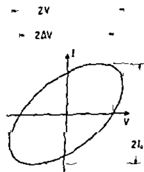


Fig 2

Fig 1 Schematic diagram of apparatus Alternating current from an oscillator (Osc) was passed through the skin (s) *via* disc electrodes (c c) The voltage across the skin was measured *via* Ringer agar salt bridges (p p) and calomel half cells (not shown) A Tektronix 502 oscilloscope (CRO) was used to measure current and voltage

Fig 2 Graph of current I against voltage V on the oscilloscope screen The quantities 2V, 2ΔV and $2I_0$ were measured directly from the screen with the aid of a graticule ruled with a 1-cm grid

TABLE I Composition of Ringer solutions (mM)

	Chloride Ringer	Sulphate Ringer
Na^+	113 6	113 6
K^+	2	2
Ca^{++}	1	1
HCO_3^-	2 4	2 4
Cl^-	115 2	—
SO_4^{--}	—	57 6

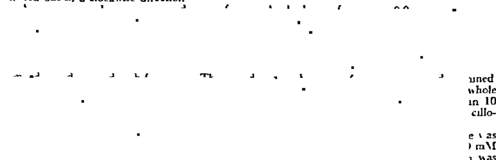
For a circuit containing both resistive and reactive components the impedance Z can be written as

$$Z = R + jX$$

where R and X are respectively the resistive and reactive components and $j = \sqrt{-1}$ is the imaginary operator R and X were calculated from the equations

$$R = [(2V_0)^2 - (2\Delta V)^2]^{1/2}/2I_0$$

and $-X = 2\Delta V/2I_0$ the measured quantities being defined in Fig 2 For a capacitative reactance X is negative, thus with the polarity of the connections shown in Fig 1, the ellipse was traced out in a clockwise direction



carried out Ringer solution was then replaced and a final run performed after a further 90 min In each experiment either chloride or sulphate Ringer was used throughout

The measurements were corrected for errors caused by the polarisation impedance of the current electrodes the error being determined in experiments with no skin present When using bare platinum electrodes the error became rather large at low frequencies with silver silver chl ted with pla

lloscope at ±1 %

and the reactive component X with the same absolute accuracy At higher frequencies (≥ 500 Hz) electrical interference limited the accuracy in both R and X to ±5 Ωcm²

Results

The results of each experimental run are expressed as a graph of −X against R—the impedance diagram As the frequency is changed the concomitant changes in X and R trace out the impedance locus of the system The impedance of a biological membrane can be represented by a resistance r and a capacitance C in parallel (Fig 3) For this circuit the values of R and X are (*e g* Cole 1968)



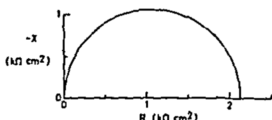


Fig 3 Equivalent circuit of a biological membrane, and the corresponding impe dance diagram (drawn for $r = 2130\ \Omega cm^2$

Na_2SO_4 Ringer both sides

100
10
1 Hz

Na_2SO_4 Ringer outside
$1/2\ Na_2SO_4$ Ringer inside

X
(kΩ cm²)

100
10
1
.01 Hz

Na_2SO_4 Ringer both sides

100
10
1 Hz

R (kΩ cm²)

Fig 4 Impedance diagrams of frog skin (date 17/7), in chronological order For the middle graph, the solution bathing the inner surface was 1/2 Ringer + 1/2 isosmotic sucrose solution Semicircular arcs were fitted by eye to the high frequency points, and to the low frequency points in the middle graph

In these equations ω (sec^{-1}) is the angular frequency of the alternating current equal to $2\pi F$, where F is the frequency, and τ is the time constant of the circuit. Equations (1) and (2) lead to a semicircular impedance locus with its centre on the R axis (Fig. 3).

An experimental impedance diagram for a skin bathed with sulphate Ringer on both sides is shown on the upper graph of Fig. 4. At frequencies above 10 Hz the points lie close to a semicircle. However there are two respects in which the experimental locus differs from the theoretical: the semicircle is shifted to the right along the R axis and the centre of the semi-circle is below the R axis. The former difference is explained by *the presence of a small resistance in series with the circuit of Fig. 3*, it can be shown that the effect of this is simply to shift the locus to the right along the R axis by an amount equal to the value of the series resistance. The depression of the centre of the semi circle below the R axis has been observed in many biological systems but has yet to receive a convincing explanation (Cole 1965).

From the experimental locus the values of r and C appropriate to the model circuit of Fig. 3 can be calculated using the equations

$$r = R_\infty - R_1 \tag{3}$$

and

$$C = 1/2\pi f\,(R_\infty - R_1) \tag{4}$$

(*e.g.* Cole 1965). R_1, R_∞ and the characteristic frequency f are defined in Fig. 5. Depression of the centre of the semicircle below the R axis is characterised by the angle φ (Fig. 5). Values of r and C for the high frequency loci obtained experimentally are given in Table II under the headings r_0 and C_0.

The values of r and C given in Table II are similar to those found by Brown and Kastella (1965), it appears that their results were determined by the properties of

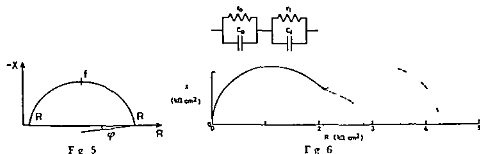


Fig. 5 Fig. 6

Fig. 5 The type of impedance diagram often observed for a biological membrane.

Fig. 6 Equivalent circuit of frog skin with the outer membrane on the left and the corresponding impedance diagram. For all these loci $r_0 = 2130\ \Omega cm^2$, $C_0 = 1.97\ \mu F\ cm^{-2}$, $\tau_0 = 4.2$ msec, $C = 50\ \mu F\ cm^{-2}$.

— — — — — $r = 2130\ \Omega cm^2$ ($r/r_0 = 1$, $\tau/\tau_0 = 36$)
— — $r = 710\ \Omega cm^2$ ($r/r_0 = 1/3$, $\tau/\tau_0 = 12$)
$r = 355\ \Omega cm^2$ ($r/r_0 = 1/6$, $\tau/\tau_0 = 6$)

the outward facing membrane of the epithelium It is therefore concluded that this *part of the locus at frequencies higher than approximately 10 Hz* represents the behaviour of the outward facing membrane

At frequencies below 10 Hz the experimental locus shown in the upper graph of Fig 4 deviates markedly from the semicircle It appeared that this deviation might be due to the inward facing membrane If the behaviour of the skin could be described by a series arrangement of two cell membranes as was proposed by Koefoed Johnsen and Ussing (1958) the skin impedance would be represented by a series combination of two circuits of the type shown in Fig 3 (Fig 6) For such a circuit the total resistive and reactive components R and X respectively are given by

$$R = R_o + R_i = r_o/(1+\omega^2\tau_o^2) + r_i/(1+\omega^2\tau_i^2) \qquad (5)$$

$$-X = -(X_o + X_i) = \omega r_o\tau_o/(1+\omega^2\tau_o^2) + \omega r_i\tau_i/(1+\omega^2\tau_i^2) \qquad (6)$$

where $\tau_o = r_oC_o$

and $\tau_i = r_iC_i$

R_o and R_i are the resistive components of the impedance of the outer and inner membranes respectively and X_o and X_i are the reactive components the other parameters are defined in Fig 6 If τ_i is much larger than τ_o the impedance locus becomes two semicircles the high frequency locus occurs as before and a second semicircular arc of diameter r_i and characteristic frequency f_i ($= 1/2\pi\tau_i$) appears at low frequencies If the difference between τ_o and τ_i is not so great there is overlap between the two semicircular arcs (Fig 6)

A better separation of the high frequency and low frequency loci is obtained if the value of r_i the resistance of the inner membrane is increased This increases both the difference between τ_o and τ_i and the diameter of the low frequency locus Such an increase in r_i was accomplished experimentally by diluting the solution bathing the inner face of the skin using isosmotic sucrose solution as diluent Under these conditions it would be expected that the numbers of ions available for carrying current *through the inner membrane would be diminished* and the resistance of this membrane thus increased The impedance locus is shown in the middle graph of Fig 4 The graph shows an increase in the size of the low frequency locus and in the separation of the two loci as predicted Upon reverting to Ringer as the inner solution the low frequency locus decreased to its previous size (Fig 4 lower graph)

When the same procedure of dilution was carried out *in the outer solution* no increase in the magnitude of the low frequency deviation occurred (Fig 7) It therefore appears that the low frequency part of the impedance locus is indeed representative of the inner membrane

Values of resistance and capacitance for the outer and inner membranes were determined from graphs of the type shown in Fig 4 and 7 A semicircle was fitted by eye to the points at higher frequencies The value of r_o was taken as the distance between the two intersections of this semicircle with the R axis (equation (3) and the value of f_o the characteristic frequency of the outer membrane was determined

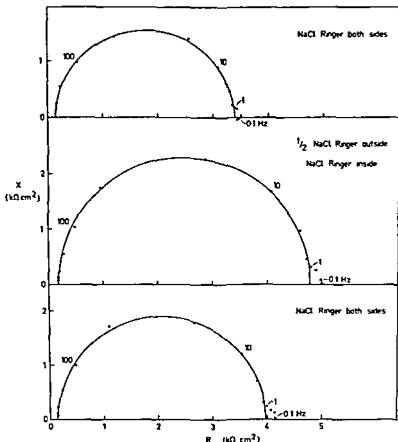


Fig 7 Impedance diagrams of frog skin (date 19/6), in chronological order For the middle graph, the solution bathing the outer surface of the skin was 1/2 Ringer + 1/2 isosmotic sucrose solution Semicircular arcs were fitted by eye to the high frequency points

by interpolation C_o was then calculated from equation (4) The values of the series resistance R_1 and the angle φ for the outer membrane (φ_o) were also recorded

The resistive impedance components of the two membranes are additive and this also applies to the reactive components (equations (5) and (6)) Therefore at the lower frequencies it was possible to subtract from the total impedance the impedance components of the outer membrane, to give those of the inner membrane The outer membrane impedance at low frequencies was calculated from the values of r_o and C_o This gave the values of R_o and $-X_o$ at each of the lower frequencies, these values were subtracted from the experimental values of R and $-X$ at the same frequencies, to give R_i and $-X_i$ A graph of $-X_i$ against R_i was then plotted and the values of r_i C_i and φ_i determined as for the high-frequency locus It was often found that the fitted locus had one negative intercept with the R_i axis, this point is considered further below

TABLE II Resistance and capacitance of outer and inner membranes of frog skin r_o and r_i are the resistances of the outer and inner membranes respectively, and C_o and C_i are the corresponding capacitances Values of parameters obtained with Ringer solution on both sides of the skin are averages of values before and after the period when the solution on one side was diluted with isosmotic sucrose solution

Solution	Date	*Ringer both sides*			*Diluted Ringer on one side*				
		pd mV	r_o Ωcm²	C_o μFcm⁻²	pd mV	r_o Ωcm²	C_o μFcm⁻²	r_i Ωcm²	C_i μFcm⁻²
Chloride	7/5	70	1000	1 59	68	1240	1 64	1220	118
Ringer	8/5	94	2130	1 72	76	3980	1 74	4470	55
inside	9 5	82	1470	1 74	45	1400	1 70	1550	93
dilution	2,6	95	1470	1 81	72	1420	1 87	2130	83
	6 6	100	4960	1 78	79	6320	1 62	4680	94
	23/6	128	2690	1 55	90	3570	1 59	3760	55
	Mean	97	2290	1 70	72	2990	1 69	2970	83
Sulphate	10/7	131	3600	1 29	133	3620	1 30	2130	49
Ringer	11/7	92	4600	1 56	89	3480	1 62	1490	89
inside	*17/7*	*93*	*1950*	*1 32*	*80*	*2540*	*1 33*	*3510*	*62*
dilution	Mean	106	3380	1 39	101	3210	1 42	2380	67
Chloride	11/6	54	1440	1 40	64	1160	1 61		
Ringer	19 6	114	3540	1 42	124	4600	1 38		
outside	24/6	110	1320	1 57	119	1160	1 80		
dilution	Mean	93	2100	1 46	102	2310	1 60		
Sulphate	12/6	58	1730	1 61	75	1780	1 62		
Ringer	14/7	139	4140	1 50	146	3420	1 41		
outside	16/7	107	5630	1 55	126	6160	1 54		
dilution	18/7	112	4520	1 50	126	4480	1 32		
	Mean	104	4000	1 54	118	4040	1 47		
	Mean of all experiments			1 56			1 57		78

The results are summarised in Table II In experiments with Ringer solution bathing the inner surface of the skin, a low-frequency deviation was seen in only about one half of the experiments and it was small in all of these The values of r_i and C_i determined from these experiments were therefore rather inaccurate and are not given in Table II However it should be mentioned that in every case the value of C_i determined from such experiments was considerably larger than that found in experiments where diluted Ringer solution bathed the inside This point is also discussed below

The low-frequency deviation became large in all experiments in which the ionic concentration of the inner solution was reduced It can be seen from Table II that experiments using chloride and sulphate Ringer give essentially the same results The capacitance of the inner membrane is approximately 50 times larger than that of the outer membrane

Values of R_1, the series resistance, determined from the impedance diagrams included the resistance of the layer of solution between the potential electrode and the skin on either side The value of the solution resistance (85 Ωcm², in Ringer solu-

tion) was determined in experiments with no skin present, and was subtracted from the total R_1 to give the contribution of the skin. This had a typical value of 35 Ωcm^2, in all solutions—a resistance much smaller than that of either membrane.

The value of φ_o did not vary with solution composition, the average value was $4.5° \pm 2.5°$ (S.D.). The value of φ_i was more variable, values ranged from 0° to 18°, with a mean of 8°.

Discussion

The experiments described here show that under certain conditions, the variation of the impedance of frog skin with frequency can be divided into two components. The high frequency component is always present, and corresponds to that observed by other workers (Teorell 1949, Brown and Kastella 1965, Cuthbert and Painter 1969). Brown and Kastella found that the resistance represented by this component was increased about twofold by complete substitution of either sodium or chloride in the outer solution by an impermeant ion, but that such changes in the composition of the inner solution had a much smaller effect. Thus it can be concluded that the characteristics of the high frequency locus are determined by the properties of the outer membrane. In the present experiments, reduction of the ionic concentrations in the inner solution had some effect on r_o, changes in the composition of the outer solution also affected r_o, but to a small extent only. Brown and Kastella found that the variation or r_o with the sodium concentration at the outer face of the skin showed a "saturation" effect, the maximum conductance occurring at a concentration of about 25 mM, conductance increased linearly with external chloride concentration. From their results it can be seen that a reduction of external ion concentrations by a factor 2 is expected to have little effect on r_o, the present results are therefore in agreement with those of Brown and Kastella.

The presence of a deviation from the semicircular impedance locus at low frequencies has not been reported before. In the experiments of Teorell (1947) and Cuthbert and Painter (1969) frequencies as low as those at which the deviation becomes apparent (below ca. 10 Hz) were not used, but the measurements of Brown and Kastella (1965) extended down to a frequency of 1 Hz. When Ringer solution bathed both sides of the skin a deviation from the semicircular locus was apparent in about one half of the present experiments. In order to investigate the inconsistency in the deviation's appearance, calculations were made of the impedance locus expected from the model circuit shown in Fig. 6. Calculations were made according to equations (5) and (6); the results are shown as impedance diagrams (Fig. 6). From these diagrams it can be seen that when the resistance of the inner membrane is low ($r_i/r_o = 1/6$), the low frequency deviation becomes indistinguishable from the high-frequency locus. It therefore seems likely that in those experiments with no apparent low frequency deviation, both in the present work and in that of Brown and Kastella, the value of r_i was much lower than that of r_o. When diluted Ringer (1/2 Ringer + 1/2 isosmotic sucrose) bathed the inner surface of the skin, the resistances of the

TABLE III True and calculated parameters of the model circuit (Fig 6) for three values of the inner membrane resistance r_i. Values of the parameters used in the construction of the impedance diagrams are on the left, values calculated from the diagrams (Fig 6), using the method described for the experimental results are on the right

True				Calculated			
r_o Ωcm²	C_o μFcm⁻²	r_i Ωcm²	C_i μFcm⁻²	r_o Ωcm²	C_o μFcm⁻²	r_i Ωcm²	C_i μFcm⁻²
2130	1 97	2130	70	2300	1 92	2100	69
2130	1 97	710	70	2330	1 96	630	83
2130	1 97	355	70	2480	1 92	(not measurable)	

inner and outer membranes were similar (Table II) It therefore appears that reduction in ionic concentrations by a factor 2 increased the resistance by a much larger factor The reason for this is not clear

From the impedance diagrams of Fig 6 values of r C and φ for both outer and inner membranes were determined using the same methods as for the experimental results, the values obtained were then compared with those used in the construction of the diagrams The results are summarised in Table III It can be seen that the method used for determining parameters from the impedance diagrams introduces inaccuracies, the parameters relating to the outer membrane (high-frequency locus) are not greatly affected but if r_i is as low as 710 Ωcm² ($r_i/r_o = 1/3$) its value is underestimated and the value of C is overestimated This effect is the most likely explanation for the large values of C_i observed experimentally when Ringer solution bathed both sides of the skin The graph of $-X_i$ for the model circuits had one negative intercept on the R_i axis indicating that this phenomenon when observed for the experimental results was not a real effect but was introduced by the procedure used for separating the two loci. Furthermore the calculated high-frequency loci were best fitted by semicircles with their centres below the R axis however the values of φ_o obtained (1 or 2 in most cases were much smaller than those seen in the experimental loci and the effect goes but a small way to explain the presence of φ

It must therefore be concluded that the method used for calculating the parameters relating to the inner membrane is not entirely satisfactory but when $r_i/r \sim 1$ the error introduced into the value of C_i is negligible (Table III) In most of the experiments from which values of C_i were calculated r_i/r was close to unity and so no errors were introduced

Brown and Kastella (1965) found values of the series resistance R_1 ranging from 50 to 190 Ωcm² in Ringer solution rather larger than the value of 35 Ωcm found in the present work This disparity could be due to a species difference Brown and Kastella used skins of *R pipiens* whereas *R temporaria* was used in the experiments reported here

[illegible] the inward facing and [illegible] was clamped between [illegible] was allowed to hang [illegible] was claimed that an electrode placed in this solution was in contact with the interiors of the epithelial cells, that is the region between the inward- and outward facing membranes It was found, using this method that the time constants of the two membranes were equal—in contrast to the results of the present work, where a large difference between the two time constants was found

The main result of the present work is that the capacitance of the inner membrane ca 78 μF cm^{-2}, is very much greater than that of the outer membrane An attempt can now be made to relate this finding to the morphology of the skin (Farquhar and Palade 1964, 1965, 1966, Voûte 1963, Voûte and Ussing 1968)

The frog skin epithelium has about five living cell layers The cells in the outermost layer are joined to one another at their apical borders by tight junctions, or zonulae occludentes, these junctions close off the intercellular spaces from the solution bathing the outer surface of the skin The outward facing membrane of the skin therefore consists of the apical membranes of the outermost layer of living cells These membranes are slightly corrugated so that the area of the outward facing membrane is perhaps 1 3 times greater than that of the skin The capacitance of this membrane (1 56 μF per cm^2 skin) is therefore ca 1 2 μF per cm^2 membrane

The mean value of the capacitance of the inward-facing membrane is 78 μF per cm^2 skin If the capacitance of the outer membrane is taken as 1 2 μF per cm^2 membrane and it is assumed that the outer and inner membranes have the same capacitance per unit membrane area, it is seen that the area of the inner membrane is about 65 times greater than the area of the skin This large membrane area puts certain limitations upon the morphological identification of the inward facing membrane

The spaces between the epithelial cells appear to communicate freely with the solution bathing the inner surface of the skin The interiors of the epithelial cells apparently communicate with one another through intercellular bridges, or desmosomes If these bridges had a low electrical resistance the functional inward facing membrane of the skin would comprise all cell membranes in contact with the inner solution, from the skin morphology it is possible to estimate the factor by which the area of this membrane exceeds that of the skin The cells are roughly cubical and therefore have a surface area 6 times greater than that of a single face this gives a 6-fold increase in area The epithelium has about 5 cell layers giving a further 5-fold increase and a total increase of 30 times In electronmicrographs it can be seen that the membranes have infoldings giving perhaps a 2 fold increase in area A total factor 60 is therefore obtained The large area of the inward facing membrane deduced from the experiments is thus accounted for on the assumption that the intercellular bridges have a low resistance

It is suggested therefore that there are bridges of low electrical resistance between the cells in the frog skin epithelium Evidence for such connections between cells has been presented by Loewenstein (1966) in several types of epithelial tissue,

TABLE III True and calculated parameters of the model circuit (Fig 6) for three values of the inner membrane resistance r_i Values of the parameters used in the construction of the impedance diagrams are on the left, values calculated from the diagrams (Fig 6), using the method described for the experimental results, are on the right

True				Calculated			
r_o Ωcm^2	C_o $\mu F cm^{-2}$	r_i Ωcm^2	C_i $\mu F cm^{-2}$	r_o Ωcm^2	C_o $\mu F cm^{-2}$	r_i Ωcm^2	C_i $\mu F cm^{-2}$
2130	1 97	2130	70	2300	1 92	2100	69
2130	1 97	710	70	2330	1 96	630	85
2130	1 97	355	70	2180	1 95	(not measurable)	

inner and outer membranes were similar (Table II) It therefore appears that reduction in ionic concentrations by a factor 2 increased the resistance by a much larger factor The reason for this is not clear

From the impedance diagrams of Fig 6 values of r, C and φ for both outer and inner membranes were determined using the same methods as for the experimental results, the values obtained were then compared with those used in the construction of the diagrams The results are summarised in Table III It can be seen that the method used for determining parameters from the impedance diagrams introduces inaccuracies, the parameters relating to the outer membrane (high-frequency locus) are not greatly affected but if r_i is as low as 710 Ωcm^2 ($r_i/r_o = 1/3$) its value is underestimated and the value of C_i is overestimated This effect is the most likely explanation for the large values of C_i observed experimentally when Ringer solution bathed both sides of the skin The graph of $-X_i$ for the model circuits had one negative intercept on the R_i axis indicating that this phenomenon when observed for the experimental results was not a real effect but was introduced by the procedure used for separating the two loci Furthermore the calculated high-frequency loci were best fitted by semicircles with their centres below the R axis, however the values of φ_o obtained (1° or 2° in most cases) were much smaller than those seen in the experimental loci, and the effect goes but a small way to explain the presence of φ_o

It must therefore be concluded that the method used for calculating the parameters relating to the inner membrane is not entirely satisfactory, but when $r_i/r_o = 1$, the error introduced into the value of C_i is negligible (Table III) In most of the experiments from which values of C_i were calculated r_i/r_o was close to unity, and so no errors were introduced

Brown and Kastella (1965) found values of the series resistance R_1 ranging from 50 to 190 Ωcm^2 in Ringer solution rather larger than the value of 35 Ωcm^2 found in the present work This disparity could be due to a species difference; Brown and Kastella used skins of *R pipiens*, whereas *R temporaria* was used in the experiments reported here

Acta physiol scand. 1971. 81. 367—375

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Histamine Release from Rat Peritoneal Mast Cells and Cat Paws Induced by Some Neuromuscular Blocking Agents

By

M Frisk Holmberg and K Strandberg

Received 31 August 1970

Abstract

Frisk Holmberg, M and K Strandberg *Histamine release from rat peritoneal mast cells and cat paws induced by some neuromuscular blocking agents* Acta physiol scand 1971 *81* 367—375

The capacity of some neuromuscular blocking agents to release histamine from rat peritoneal mast cells and cat paws has been investigated Only nondepolarizing agents were effective in the ... dimethyltubocurarine ... nding order of neuro- ... and dimethyltubocu ... fused cat paws The ... by potassium cyanide and lack of Ca^{++} It is concluded that the interonium distance in neuromuscular blocking agents may be critical for histamine releasing capacity

It is well established that the release of histamine from rat and cat tissues induced by polymeric compounds *e g* the synthetic polyamine compound 48/80 is dependent on intact energy yielding enzymatic reactions (Uvnäs 1968) Tubocurarine a quaternary ammonium compound has recently been shown to release histamine from rat peritoneal mast cells (Frisk Holmberg and Uvnäs 1969) and in cat paws (Strandberg 1971 b) by a mechanism similar to that activated by compound 48/80 In addition to histamine there was an efflux of slow reacting substance (SRS) from the cat paw perfused with tubocurarine

In the present work the property of some other potent neuromuscular blocking agents nondepolarizing as well as depolarizing drugs (see Fig 1) to release histamine from rat peritoneal mast cells has been studied and compared with the effects of tubocurarine Effective compounds were also studied on cat paws

Experimental procedures

Preparation of cells and incubation technique

Male Sprague Dawley rats (350—400 g) were lightly anesthetized with ether and bled by cutting the carotids 9 ml of isotonic salt solution (pH 6 8 NaCl 154 mM KCl 2.7 mM $CaCl_2$

1 0 mM) containing 10 % (v/v) Sörensen buffer ($Na_2HPO_4 + KH_2PO_4$ 67 mM) was injected into the abdomen The cell suspension was collected with a pasteur pipette and put into plastic tubes After 2 washes with salt solution *vide supra* the cells were suspended in salt solution pH 7 2 containing 0 05 % human serum albumin and incubated for 10 min at 37° C with liberator, thereafter immediately cooled and centrifuged 350×g at 4° C for 10 min After decanting, 1/10 volume of 1 N HCl was added to both supernatant and sediment

In the Ca^{++} studies, the cell suspension was prepared and washed three times in the same medium as above but Sorensen phosphate buffer was exchanged for 50 mM Tris buffer pH 7 2 and Ca^{++} and human serum albumin were omitted Incubation was performed with or without Ca^{++} 1—2 mM added to the incubation fluid prior to exposure to liberator

Peritoneal cells suspensions contain 3—5 % mast cells and it has been shown that these cells are the only histamine containing structures (Smith 1958) Therefore, isolation of the mast cells was not regarded as essential for the present study

Cat paw perfusion

After arterial cannulation the paws were perfused with a salt solution (NaCl 154 mM, KCl 2 7 mM $CaCl_2$ 0 9 mM containing 10 % (v/v) Sörensen's phosphate buffer (Na_2HPO_4+ KH_2PO_4 67 mM), pH 7 0, until the effluents were visibly free from blood Then the paws were mounted in temperature controlled chambers (27° C) and perfused using constant rate (1 ml/min) infusion pumps (Strandberg 1971 a)

The spontaneous efflux of histamine and SRS into the perfusate was estimated for 20 min prior to addition of releaser The effluents were collected during 20 min periods in *calibrated glass tubes cooled by ice water*

The perfusates were centrifuged 350×g at 4° C for 10 min to remove any blood cells The supernatants were briefly boiled and centrifuged From each supernatant two 0 5 ml samples were removed for histamine assay, the rest of the supernatant was used for assay of SRS

When the influence of potassium cyanide on the efflux of histamine and SRS was studied, it was added in corresponding amounts to effluents from paws not perfused with inhibitor In the experiments with Ca^{+} 50 mM Tris buffer was used instead of the Sörensen buffer and Ca^{+} was omitted from the basic perfusion medium

Assay of histamine

Determination of histamine was done fluorimetrically according to Shore *et al* (1959) using a Farrand fluorometer model A 3

Assay of SRS

SRS in the effluents was determined by bioassay using the guinea pig ileum as described by Chakravarty (1959) The smooth muscle preparation was suspended in a 4 ml organ bath containing aerated Tyrode solution (37° C) with atropine sulphate (1 μg/ml) and mepyramine maleate (1 μg/ml) SRS activity is expressed in SRS-units referring to a standard of cat paw SRS (Strandberg 1971 a)

None of the compounds in the concentrations used interfered with the determination of histamine or SRS

Materials

ents were started Tosi
by Hoffman La Roche,
Chem comp St Louis
iethonium bromide and
serum albumin was ob-

Results

Dose-effect relationships

Upon exposure to 10⁻⁴—5 10⁻³ M of the drugs used rat mast cells released increasing amounts of histamine (see Table I) The approximate potency for histamine release on equimolar basis was for dimethyltubocurarine toxiferine-C and alcuronium, one half, one third and one fifth respectively compared to that of tubo-

TABLE I Release of histamine from rat peritoneal mast cells by neuromuscular blocking agents

DRUG	10^{-4}	$5 \cdot 10^{-4}$	10^{-3}	$5 \cdot 10^{-3}$ M
Tubocurarine	17 (13—20)	42 (36—45)	57 (53—65)	64 (62—67)
Dimethyltubocurarine	8 (7—11)	20 (17—21)	33 (31—35)	37 (35—39)
Alcuronium	2 (1—3)	6 (5—8)	15 (13—17)	20 (17—22)
Toxiferine C	5 (4—6)	14 (12—15)	24 (22—27)	29 (27—31)
Gallamine	—	10 (9—11)	11 (11—12)	11 (9—13)
Decamethonium	—	3 (2—6)	4 (3—7)	10 (8—13)
Suxamethonium	—	5 (4—5)	5 (4—6)	15 (14—15)

The figures represent percentage release of total histamine content, mean and range of 3—5 experiments carried out with duplicate samples, spontaneous release deducted (less than 1 %)

curarine The depolarizing drugs and gallamine had practically no effect For this reason only the histamine and SRS releasing properties of tubocurarine dimethyltubocurarine, toxiferine C and alcuronium were investigated in cat paws The drug concentration used was $5 \cdot 10^{-4}$ M because in cat paws this concentration of tubocurarine always produces release of histamine and SRS (Strandberg 1971 b) In our study besides tubocurarine only dimethyltubocurarine was effective, tubocurarine being the more potent of the two (Fig 2) No quantitative correlation between the appearance of histamine and formation of SRS was found

NONDEPOLARIZING AGENTS

	R
TUBOCURARINE	H
DIMETHYL TC	CH_3

	R
C TOXIFERINE	CH_3
ALCURONIUM	$CH_2CH{=}CH_2$

GALLAMINE

DEPOLARIZING AGENTS

$(CH_3)_3N^+(CH_2)_{10}N^+(CH_3)_3$ DECAMETHONIUM

SUXAMETHONIUM

Fig 1 Structural formulas for neuromuscular drugs used

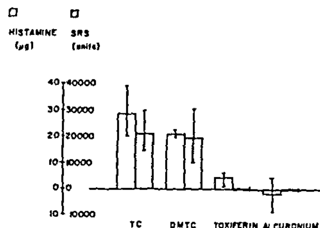


Fig 2 Efflux of histamine and SRS from cat paws perfused with tubocurarine (TC) dimethyl tubocurarine (DMTC) toxiferine C and alcuronium (5×10^{-4} M) for 80 min at 27° C. Each column represents the mean of 3 experiments, vertical bars denote ranges Estimated spontaneous release deducted

Effect of potassium cyanide and Ca''

Potassium cyanide and Ca'' lack inhibit histamine and SRS release in cat paws perfused with tubocurarine (Strandberg 1971 b) *In the present study the influence* of potassium cyanide and Ca lack on the release processes in rat mast cells as well as in cat paws was investigated Since the histamine and SRS releasing effects of toxiferine-C and alcuronium were very low in both species only tubocurarine and dimethyltubocurarine were used as releasers

Histamine release caused by tubocurarine and dimethyltubocurarine was diminished when the rat mast cells were pre-incubated for 10 minutes with potassium cyanide in concentrations 10^{-5}—10^{-3} M prior to addition of the releasing agent (Fig 3)

Potassium cyanide inhibited the release of histamine and that of SRS in the cat paw, whether induced by tubocurarine or dimethyltubocurarine A representative experiment is shown in Fig 4 In these experiments the inhibitor was administered also during the 20-min period preceding the introduction of the releasing agents In addition, the illustrated experiments demonstrate that regardless of releaser the efflux of SRS was delayed compared to that of histamine

The release of histamine from rat mast cells exposed to tubocurarine and dimethyltubocurarine, 10^{-3} M in media containing 10^{-3} M Ca was 54 per cent and 30 per cent higher than the release in a Ca''-free medium (Fig 5) Doubled Ca'' concentration did not increase the release further

In cat paws both neuromuscular blocking agents evoked an efflux of histamine and SRS in the absence of Ca Less histamine and SRS appeared in the effluents from paws receiving disodium-EDTA 2 10^{-3} M (Fig 6) The results in two other experiments were similar Subsequent administration of the releasing agents together with Ca'' resulted in an increased efflux of histamine and SRS from paws treated with disodium-EDTA and usually also although less pronounced from the other paws

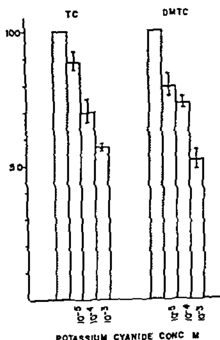


Fig 3 Effect on histamine release by pretreating rat mast cells with different concentrations of potassium cyanide before exposure to tubocurarine (TC) and dimethyltubocurarine (DMTC) (10^{-3} M) Unfilled bars denote samples pretreated with inhibitors Filled bars represent controls i e samples only exposed to liberator Spontaneous release deducted Means and ranges of 2 experiments with duplicate samples

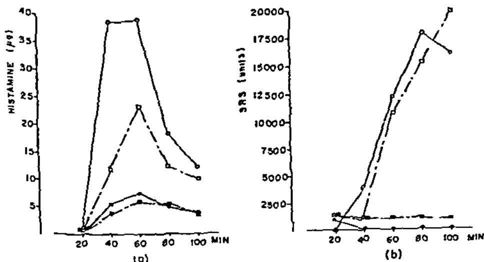


Fig 4 Influence of potassium cyanide (10^{-4} M) on the efflux of histamine (a) and SRS (b) from cat paws perfused with tubocurarine (5×10^{-4} M) (circles) or dimethyltubocurarine (5×10^{-4} M) (squares) Filled symbols denote efflux from paws perfused with potassium cyanide as well

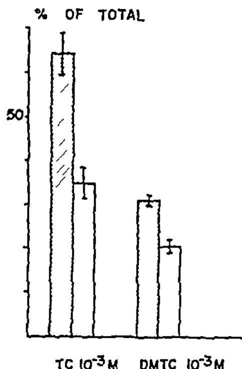
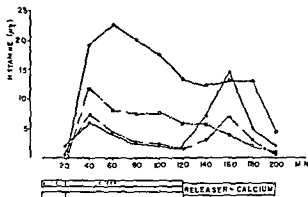


Fig 5 Effect of Ca on the release of ... ramine ... (TC) ... bated ... cells incubated with 10^{-3} M Ca Spontaneous release in Ca free media and in media containing 10^{-3} M Ca was 6 % and 5 % respectively Means and ranges of three experiments carried out with triplicate samples

Discussion

The present investigation has shown that upon exposure to tubocurarine dimethyl tubocurarine toxiferine C and alcuronium rat mast cells and cat paws respond in essentially the same manner with regard to histamine release Of the drugs studied and compared to tubocurarine dimethyltubocurarine turned out to be most potent having about half the activity of tubocurarine on an equimolar basis Toxiferine-C and alcuronium had very low histamine releasing activity and gallamine suxamethonium decamethonium were ineffective in the concentration range used Histamine release in cat paws induced by tubocurarine or dimethyltubocurarine was in all experiments accompanied by an efflux of SRS

In both species histamine release on exposure to tubocurarine has been shown to be reduced by metabolic inhibitors (Frisk Holmberg and Uvnas 1969 Strandberg 1971 b) In agreement with these findings potassium cyanide an inhibitor of cell metabolism diminished the release in the present study This inhibitor was selected since it has earlier been demonstrated to inhibit histamine release from cat paws caused by tubocurarine (Strandberg 1971 b) It has recently been shown that histamine release from cat paws caused by tubocurarine is Ca dependent (Strandberg 1971 b) This was confirmed in the present experiments and it was also shown that the histamine release from rat mast cells in a Ca free medium was almost 50



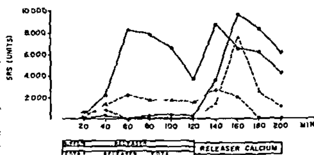


Fig 6 Influence of Ca⁺ on the efflux of histamine (a) and SRS (b) from cat paws perfused with tubocurarine (5×10^{-4} M) (circles) or dimethyltubocurarine (5×10^{-4} M) (squares) Releasers and chemicals were added to the basic Ca free perfusion medium as follows

	0—20 min	20—120 min	120—200 min
Open symbols	0	releaser	releaser + $CaCl_2$ 5×10^{-3} M
Filled symbols	Na_2EDTA 2×10^{-3} M	releaser + Na_2EDTA 2×10^{-3} M	releaser + $CaCl_2$ 5×10^{-3} M

per cent lower than the release in a medium containing 10^{-3} M Ca Dimethyltubocurarine was similarly influenced as tubocurarine by potassium cyanide and Ca indicating that both agents activate the same release mechanism Tubocurarine has been shown to release histamine from isolated rat mast cells by a similar degranulating mechanism as compound 48/80 (Frisk Holmberg and Uvnas 1969) The conception of Ca^{++} dependence regarding compound 48/80 induced histamine release is controversial Whereas histamine release from tissues induced by compound 48/80 requires Ca (Chakravarty 1960 Mota and Ishii 1960 Rotschild 1970) the degranulation and histamine release from isolated mast cells has been reported to be unchanged by Ca lack (Uvnas and Thon 1961 Saeki 1964) In our study lack of Ca^{++} reduced the release in both experimental systems used It should be pointed out however that in the present investigation a peritoneal cell suspension was used instead of isolated mast cells

The present results have thus further confirmed the hypothesis suggested by Frisk-Holmberg and Uvnäs (1969) that histamine release induced by tubocurarine is an active process dependent on intact energy metabolism. Interestingly enough histamine releasers which exert their releasing action through a change in the membrane permeability like chlorpromazine are uneffected by metabolic blockers and histamine release is increased by lack of Ca (Frisk-Holmberg 1970).

It has been suggested that clinically used nondepolarizing neuromuscular blocking agents other than tubocurarine also have histamine releasing properties. Apart from a study on the effect of alcuronium on the histamine blood levels during anesthesia in humans (where no increase in blood histamine was found, Waser and Harbeck 1962) only indirect methods of measuring histamine release seem to have been used such as wheal (Chasserant 1963, Bush 1965) and bronchoconstriction (Bächtold 1964). In those studies tubocurarine was more potent than toxiferine C and alcuronium respectively; dimethyltubocurarine was not studied. The results are however in agreement with our findings.

In our experiments free hydroxyl groups in the molecule promoted histamine release as tubocurarine was more potent than dimethyltubocurarine. The steric configuration of these compounds and the distance between N—N may be of importance for the histamine release. This suggestion is supported by the finding that the depolarizing drugs and gallamine had practically no histamine releasing property in the concentration range used in this study and it has been shown that the interonium distance is 12.5 Å for curare compounds (Goodman and Gilman 1965) and 14 Å for toxiferines (Schmid 1967). The neuromuscular blocking potency of the drugs used is in a different rank order (Waser 1966, Stenlake 1968) than their effectiveness as histamine releasers as demonstrated in the present study. In conclusion no correlation exists between the histamine releasing capacity of the drugs and their neuromuscular blocking activity in the species studied.

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Effects of Vasoconstriction on Blood Viscosity in Vivo

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Abstract

BAECKSTROM P B FOLKOW E KENDRICK B LÖFVING and B ÖBERG *Effects of vasoconstriction on blood viscosity in vivo* Acta physiol scand 1971 *81* 376—384

The influence of vascular dimensional changes on blood viscosity *in vivo* ('effective' viscosity) was analyzed in the calf muscle vascular bed of cats. Values for effective blood viscosity over an extensive flow range were obtained from comparisons of pressure flow curves for blood and a Newtonian fluid (Dextran Tyrode) with known viscosity. Such comparisons were made both at high and low flows during maximal vasodilatation and at low flows during vascular narrowing caused either by vessel collapse or by intense stable vasoconstriction as a result of barium chloride infusion. At indentical low flows effective blood viscosity was substantially lower in the constricted or collapsed bed than in the dilated and distended bed. With reduction of flow from maximal to very low values in the completely dilated non collapsed bed effective blood viscosity increased from about 2.35 cP by 200—300 per cent. If the same low flows occurred as a result of intense vasoconstriction implying a more than 5000 per cent increase of flow resistance the viscosity increased by about 30 per cent.

Apparently effective blood viscosity is normally low and varies very little within the extensive flow range met with *in vivo* as a consequence of alterations of vascular tone. Therefore even at extremely low flows in the constricted bed as after severe blood loss effective blood viscosity is hardly a significant determinant of flow as long as blood composition is not changed.

In a recent study (Djojosugito *et al* 1970) blood viscosity *in vivo* ('effective viscosity) and its dependence on flow rate was measured in cats utilizing the maximally dilated calf muscle bed as the biological viscometer. This study showed that effective blood viscosity was throughout low being at high flow rates about 2.35 cP or some 50 per cent of the value obtained on the same blood with *in vitro* measurements at high shear rates in a Wells Brookfield viscometer. With reductions of flow rate, effective blood viscosity increased by approximately 100 per cent when blood flow was reduced about 100 times but at the very lowest flows it tended to decrease

[1] Supported by a special research fellowship (1FO3He05322) from the National Institutes of Health National Heart Institute. Present address Department of Physiology University of Wisconsin Madison Wisconsin USA

again Thus the very steep increase of blood viscosity found in *in vitro* systems at very low shear rates had apparently no counterpart in the *in vivo* situation

The decrease of effective blood viscosity at very low flows was proposed to be due to an enhanced Fåhraeus—Lindqvist effect and the emergence of so called bolus flow in some of the main resistance sections, consequent to a widespread elastic recoil and/or collapse of the microvessels and hence reduction of their dimensions If this interpreatation is correct prevention of vessel collapse would result in a more pronounced increase of effective blood viscosity at very low flows and shear rates One of the objectives of the present study was to analyze whether this was indeed the case

Flow dependent alterations of effective blood viscosity thus seem to be rather moderate What is more, the viscosity data in the earlier study mentioned were obtained in a maximally dilated bed, thus with especially large vessel dimensions also at low flows This situation will tend to increase effective blood viscosity because of the relatively low linear flow velocity and shear rate and a less effectively operating Fåhraeus Lindqvist mechanism in the widened vessels Reductions of blood flow are however, normally produced by a reduction of vessel dimensions *i e* by vasoconstriction rather than by a reduction of perfusion pressure as was the case in the study mentioned above It is therefore reasonable to assume that flow dependent alterations of effective blood viscosity are still smaller in the normal situation where alterations of flow are caused by shifts in vascular tone The second aim of the present study was to explore whether this assumption was correct — A brief summary of the present results have been presented earlier (Baeckstrom *et al* 1970)

Methods

Experiments were performed on cats anaesthetized with chloralose 40—50 mg/kg b w Preparation of the calf muscles was carried out as earlier described in detail (Djojosugito *et al* 1970) The muscles were completely isolated from the animal except for the cognate artery and vein Three arterial branches from the femoral artery were cannulated the most distal one to provide for measurements of arterial inflow pressure the intermediate one for drug infusions and the widest and most proximal one to provide a means of perfusing the calf muscles with an oxygenated body warm Dextran Tyrode solution (Dextran) This solution was either a mixture of 3 % Macrodex and 5 % Rheomacrodex or 5 % Rheomacrodex in Tyrode solution and displayed viscosities at 37° C of 2 9 cP and 1 8 cP respectively The perfusion of the muscles could be rapidly changed between the normal blood supply from the *animal through* the intact femoral artery and the *Dextran Tyrode reservoir*

The femoral vein was cannulated and the venous outflow passed through an optical drop recorder for continuous measurement of blood (or Dextran) flow More accurate flow determinations were made by diverting the blood *via* the drop recorder through a graduated pipette and timing the movement of the fluid meniscus between the divisions The blood was returned to the animal *via* a funnel connected to a jugular vein During Dextran perfusion the outflow from the muscles was discarded

The free proximal end of the draining venous catheter could be placed so as to set the venous outflow pressure at any desired level Venous outflow and arterial inflow pressures [illegible] -noral vein and artery [illegible] on a level with the [illegible] on a Grass Polygraph [illegible] when a steady state flow was at hand as evident from the continuous drop recording [illegible] ulation electrodes were placed on the distal end of the cut sciatic nerve to induce maximal dilatation in the preparation by means of intermittent exercise

In 5 expts the calf muscle preparation was placed in a plethysmograph (see Kjellmer 1964) for estimations of the capillary filtration coefficient (CFC) By means of such measurements the extent of "collapse" of capillaries and adjacent microvessels at very low transmural pressures could be evaluated

... n were made in a ... dilation was in di ... rge amounts of isoprenaline (10—15 μg) or papaverine (1—5 mg) Each experiment began with a determination of the pressure flow curve for blood and Dextran in the maximally dilated bed where for each given pressure head the transmural pressure was kept the same for the two perfusates From the two press ... the maximally dilated bed could be detern ... 0)

In one series of ... d Dextran were again determined during ... pressure kept elevated at approximately 2 ... ffective blood viscosity could thus be compared when a fully relaxed vascular bed was either kept well distended or allowed to collapse at the lowest pressure heads Repeated determinations of CFC were performed in these two situations to check the extent of small vessel collapse

In another series of experiments the vessels were brought into various levels of constriction by means of i a infusion of barium chloride (2—10 mg/min dissolved in 0 1—0 2 ml Dextran) during perfusion with Dextran at a pres ... vasoconstriction corresponding to about ... between blood and Dextran perfusion ... was stopped just prior to these procedu ... sustained that several measurements could be performed before the vessels relaxed again — Flows for the two perfusates at identical pressure heads and transmural pressures or the perfusion pressure at identical flows were then compared so that effective blood viscosity could be calculated at the prevailing level of regional flow resistance

To make such calculations valid it is of course very important that a *complete* shift between the two perfusates had occurred in the main resistance vessels before any measurements were performed To check this rapid shifts between perfusion with Dextran and Tyrode solution both being Newtonian fluids but with very different viscosities were made when the vessels were still maximally dilated to avoid luminal changes When after shifting the perfusion fluid flow again reached a steady state this was taken to indicate that the preceding perfusate had been completely washed out from the resistance vessels This was found to be regularly the case with less than 1 ml of inflow to a 50 g calf preparation that at maximal dilation contains roughly 1 5 ml blood most of which resides in larger veins that contribute only little to flow resistance During the influence of barium one may expect that even less inflow is needed for a complete shift since the volume of the vascular bed is now reduced Nevertheless comparisons of Dextran and blood flows were not performed until at least 1 5 ml of the new perfusate had entered the vascular bed

Results

I Effects of vessel collapse on blood viscosity in vivo

Technically successful experiments of this type were performed on 5 animals The results from these experiments are shown in Fig 1 describing the relation between the calculated values for effective blood viscosity during maximal vasodilation and the blood flow expressed as ml/min × 100 g tissue (log scale) The viscosity value for the "reference" Dextran Tyrode solution is also shown It is seen that effective blood viscosity being low at very high blood flows progressively increases as flow decreases If venous outflow pressure is kept low so that vessel collapse occurs when the transmural pressure falls along with the reduction of the pressure head effective blood viscosity increases to a maximal value when blood flow is reduced to about 1 ml/min × 100 g It is then approximately twice as high as the viscosity at the very highest flow rates When pressure head and flow are further reduced effective blood

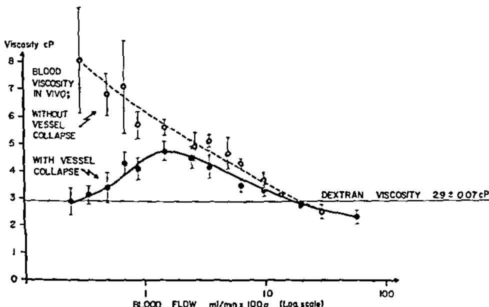


Fig 1 Effects of alterations of blood flow on effective blood viscosity in a maximally dilated vascular bed when vessel collapse occurred (solid line, filled circles) and when collapse was prevented by keeping venous outflow pressure high (broken line, open circles) The data are obtained from 5 expts The height of the vertical bars correspond to ±SE of the mean viscosity value

viscosity can be seen to decrease again, a finding confirming earlier observations (Djojosugito *et al* 1970) When CFC, reflecting the size of the perfused capillary surface area, was determined in the maximally dilated vascular bed in 5 additional experiments, it was found that CFC remained fairly constant as long as the estimated mean capillary pressure was kept above 12—14 mm Hg If however, this pressure was further reduced by a lowering of the arterial pressure, CFC fell markedly indicating that a considerable collapse and/or stagnation of capillary flow takes place in the microvascular network at very low transmural pressures

If, on the other hand the venous outflow pressure is throughout maintained high so that collapse of the small vessels is prevented at low pressure heads apparent blood viscosity increases continuously with reductions of flow (Fig 1) It is seen that under these conditions, effective blood viscosity could increase to values more than 3 times higher than those obtained at very high flow rates This steep rise of blood viscosity at low flows actually resembles that obtained in *in vitro* systems at very low shear rates These findings thus support the hypothesis that the fall in effective blood viscosity at extremely low flows in the maximally dilated bed is due to partial collapse of small resistance vessels and capillaries

II Effects of active vasoconstriction on blood viscosity

Technically successful experiments were carried out on seven cats The results from these experiments are summarized in Fig 2 The calculated values for effective blood

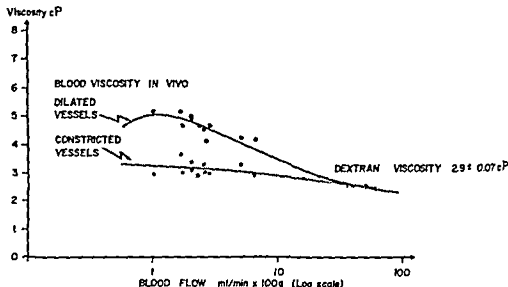


Fig. 2. Effects of alterations of blood flow on effective blood viscosity in a maximally dilated (solid line, filled symbols) and a constricted (broken line, open circles) vascular bed. Each circle represents the mean value of repeated measurements at a given level of vasoconstriction in the individual animal. The filled symbols represent viscosity values found at the same flow value in the same vascular bed when it was maximally dilated. The data are obtained from 7 expts. The mean value for effective blood viscosity *in vivo* at mean maximal flow and the range of variation of these two variables obtained in the seven experiments is also shown.

viscosity in the maximally dilated and in the constricted vascular bed, respectively, have here been plotted against blood flow (log. scale). It can be seen that the increase of blood viscosity with reduced flow rates is much less pronounced when flow reduction is caused by progressive vasoconstriction than when blood flow is reduced in the maximally dilated bed by lowering of the pressure head. Thus, with reductions of flow from high values (50—70 ml/min × 100 g) to very low values (1—3 ml/min × 100 g) blood viscosity increases only about 30 per cent in the constricted bed compared to almost 100 per cent in the collapsing maximally dilated bed and up to 300 per cent if this collapse is avoided. These data strongly suggest that flow dependent alteraions of effective blood viscosity under normal conditions where flow reductions are caused mainly by active vasoconstriction rather than by changes in the pressure head are very small indeed.

Discussion

Methodological considerations. The values for blood viscosity *in vivo*, (effective viscosity) obtained in the present experiments were calculated from comparisons of blood and Dextran flows at identical perfusion and transmural pressures in a normal vascular bed. Certain presumptions must, however, be made to justify such deductions. Thus Dextran viscosity must be regarded as largely constant irrespective of flow. That this is the case is very likely since *in vitro* measurements in different types

of viscometers and at different shear rates yield essentially identical values for Dextran at identical temperatures

It is further most important that a *complete* shift really took place between the two perfusates in the resistance vessels before any comparisons were made This was carefully checked by shifting between two Newtonian fluids at known viscosities as described in Methods and it is therefore very unlikely that such a source of error significantly affected the present results

Another all important prerequisite is that vascular dimensions are identical for blood and Dextran at each point of comparison This is most probably the case when the vascular bed is maximally dilated and the mean distending pressure kept the same at a given pressure head for the two perfusates However in those experiments where a vasoconstriction was produced by barium chloride infusion it can be more difficult to exclude with certainty that increases in vascular tone did not occur in connection with shifts from Dextran to blood perfusion and *vice versa* because of the superior nutrition furnished by the blood with a consequent accentuation of smooth muscle reactivity during blood perfusion By using vasoconstrictor agents with a more rapid onset and elimination of action *e g* noradrenaline it proved to be impossible to prevent alterations of vascular tone with shifts of perfusate

The unphysiological method of establishing smooth muscle constriction by means of barium ions was however characterized by very sluggish changes in vascular tone There was for such reasons often sufficient time to shift from one perfusion fluid to the other and back again with essentially no evidence of any significantly changed vascular tone during the measurement period In such experiments Dextran and blood flows rapidly attained a constant value after a transient wash out period upon sudden shifts of perfusate and with repeated shifts reproducible Dextran flows and blood flows could be obtained

These circumstances were taken as evidence of stable and well maintained vascular tone and dimensions not significantly influenced by the nature of perfusing fluid for the period needed for the measurements These experiments were used for deductions of effective blood viscosity during vasoconstriction and the data presented in Fig 2 are derived from such animals However even when barium chloride was utilized for the constriction there was indeed in some experiments evidence of slow but clearcut decreases of vessel tone in connection with shifts of perfusate from blood to Dextran and *vice versa* revealed as a slight but continuous increase of Dextran flow upon shift from blood perfusion and a slight but continuous reduction of blood flow when blood perfusion was reestablished despite constant pressure head throughout Such experiments were discarded in the present study It should be stressed however that such a technical error will if anything tend to give too high values for effective blood viscosity during vasoconstriction.

Evaluation of the results The results of the present study thus support the idea that the reduction of effective blood viscosity seen in the maximally dilated bed whe pressure head and flow are very low is related to a collapse of the microvessels was evident also from the considerable reduction of CFC found when bo

sion and transmural pressures were very low With such a partial obstruction of the smallest vessels their contribution to the total flow resistance will increase Consequently the regional viscosity in this vascular section which is inherently the lowest one along the vascular circuit will influence effective blood viscosity to an increasing extent

The present data further show clearly that the increase of effective blood viscosity *concomitant to a reduction of blood flow* is very small when flow is reduced by vasoconstriction i e by an active reduction of vessel dimensions which is the way in which flow is normally reduced in the intact organism On the basis of *in vitro* measurements increases of blood viscosity have however often been regarded as a most important determinant of blood flow in situations with reduced flow rates such as occurs after blood loss etc The present study of effective blood viscosity seems to refute this hypothesis as long as the composition of the blood is not changed as well An example based on data from the present study might clarify this Under normal resting conditions blood flow in skeletal muscles amounts to about 4—6 ml/min × 100 g If blood flow is reduced to 0 5—1 ml/min × 100 g due to an intense reflex vasoconstriction effective blood viscosity would increase only by some 15 per cent to judge from the present experiments Similarly when muscle blood flow increases to maximal values during exercise (50—70 ml/min × 100 g) due to a complete relaxation of vascular smooth muscles effective blood viscosity would becrease only by some 15 per cent

In other words at an almost 100 fold increase of flow resistance effective blood viscosity would increase by a factor of only 1 3 This may appear surprising considering the fact that effective viscosity increased about 3 fold when flow was equally reduced in the dilated and distended vascular bed It should however be realized that a luminal narrowing which causes a 100 fold flow reduction implies a more than 3 fold decrease in radius in an idealized resistance vessel and therefore only a 10-fold decrease of linear flow velocity In contrast an identical diminution of flow at an unchanged vascular radius implies a 100 fold reduction of linear flow velocity At identical flows shear rate is thus 10 times higher in the narrowed vessel and effective blood viscosity must for such reasons alone be lower during vasoconstriction In addition the Fåhraeus Lindqvist effect will further tend to reduce effective viscosity in this latter situation other things being equal The joint operation of these two factors known to affect the anomalous viscous properties of blood may well explain the very small rise in effective viscosity that occurred when maximal flows during varodilatation were changed into minimal ones in connection with intense vasoconstriction

In an experimental study on dogs Pappenheimer and Maes (1942) made an interesting addition to the classical investigation on blood viscosity in vivo performed by Whittaker and Winton already in 1933 in the dilated vascular bed Pappenheimer and Maes tried to go a step further by estimating the relative contribution of enhanced blood viscosity to the increase of total flow resistance that is produced by vasoconstriction They interpreted their data to indicate that increases in effective

blood viscosity might account for as much as one third of the resistance increase occurring as a result of vasoconstriction This calculated marked *increase of effective* blood viscosity during vasoconstriction can however probably be explained by uncontrollable adjustments of vascular dimensions *i e* by an augmented vasoconstriction when they shifted from the Newtonian reference fluid (in their case Ringer solution) to blood

As already mentioned such changes in vascular tone in connection with shifts of perfusate constitute a crucial and most difficult problem in experiments of this type Particularly when vasoconstriction is induced by catecholamines as was the case in Pappenheimer s and Maes study changes in vascular tone can be both rapid and extensive in connection with shifts of perfusate to judge from exploratory experiments in connection with the present study The observed increase of flow resistance when blood is used is then of course not only an expression of effective viscosity differences between blood and the reference perfusate but to an unknown and perhaps dominating extent due to vascular narrowing

In case the value given by Pappenheimer and Maes—*i e* that an increased blood viscosity might account for as much as one third of the resistance increase occurring during vasoconstriction—were generally valid effective blood viscosity would increase some 1500 per cent when muscle flow resistance increases 50 times *e g* when maximal exercise hyperaemia is transformed into moderate neurogenic vasoconstriction during rest According to the present results the actual figure for viscosity increase is then at least 50 times smaller or only some 30 per cent Expressed in another way changes in effective viscosity of normal blood appear to contribute with only a few per cent *at most to the resistance changes that are caused by altered* dimensions of the resistance vessels If so the viscosity changes are in fact so small that effective blood viscosity may almost be considered to be constant in most physiological situations provided that blood composition is not concomitantly changed

It should however be emphasized in this context that the present experiments deal with the influence of viscosity factors on b ood flow through the vascular bed as a whole *i e* with the effective viscosity of blood where only the average effect of blood flow rate and vascular dimensions on the anomalous viscous properties of the blood are taken into account It is possible that more local alterations of blood viscosity may affect other important functions of the microcirculation in a more decisive way For example particularly during very low flow rates regional blood viscosity might increase relatively more in the wide bore postcapillary venous section than in the more narrow precapillary resistance section If o this would mean that flow resistance in post capillary vascular sections may be critically elevated by the viscosity factor *per se* This again would lead to a rise of capillary pressure with a consequent disturbance of the current Starling equilibrium so as to favour filtration losses from the blood across the capillary membranes Such possible disturbances of the Starling equilibrium as a result of a viscosity dependent increase of postcapillary flow resistance will not become evident in studies of the type presently described but will be subjected to investigation in future experiments

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Acta physiol scand. 1971. 81. 385—395

From the Laboratory for the Theory of Gymnastics University of Copenhagen Denmark

The Effect of High Oxygen Tensions on Ventilation during Severe Exercise

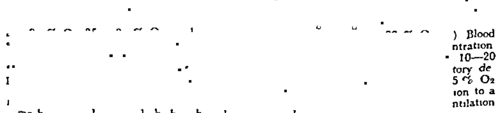
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Abstract

Kozlowski, S, B Rasmussen and W G Wilkoff *The effect of high oxygen tensions on ventilation during severe exercise* Acta physiol scand 1971 *81* 385—395



by the same factors at rest and during exercise If one assumes that the aortic and carotid chemoreceptors are responsible for the depression in ventilation the threshold value of these receptors is changed during severe exercise since 35 % O_2 is not enough to silence the chemoreceptors

The depression of ventilation produced by the inhalation of hyperoxic gas mixtures during heavy exercise has been observed by many authors and most thoroughly examined by Asmussen and Nielsen (1946 and 1958) and Bannister and Cunningham (1954) Asmussen and Nielsen interpreted their observation of a graded response to mixtures of 30 60 and 100 % O as a stepwise diminution of a work substance produced anerobically in the working muscles Bannister and Cunningham observed a graded response between 33 % and 66 % O but could not separate the effect of 66 % and 100 % O They explained their results on the basis of an assumed arterial hypoxemia and acidosis It seems that both explanations could be combined in a two

component response to oxygen breathing in which the depression of ventilation resulting from the inhalation of 30—35 % O_2 would be due to abolition of a chemoreceptor drive whereas any further increase in the inspired O_2 tension would depress ventilation via an interaction with the production of some anerobically produced metabolite To examine this possibility it was decided to study during heavy exercise the ventilatory transients produced by switching to gas mixtures of various O_2 tensions The transients were studied on a breath to breath basis, the assumption being that any respiratory transient produced by a change in chemoreceptor activity would be significantly faster than one resulting from some chemical changes involving an anerobic work substance Although a rise in P_{CO_2} and a gradual fall in lactate have been observed by both Asmussen and Nielsen and Bannister and Cunningham, these had not been correlated with the time course of the fall in ventilation It remained to be shown that these changes were a result of the change in ventilation and not causal

According to Witzleb *et al* 1955 and Dejours *et al* 1957 breathing of 35 % O_2 results in an elevation of arterial P_{O_2} sufficient to abolish chemoreceptor activity Hence the transients were to be elicited by switching the inspired O_2 tensions from air (21 % O_2) to 35 % O_2 (A 35) (chemoreceptor component), 35 % O_2 to 100 % O_2 (35—100) (metabolic component), air 100 % O_2 (both components) and 100 % O_2 to air (100 A) (off response)

Procedure and Methods

The subjects were four healthy and well trained students who were familiar with the respiratory apparatus but unaware of the nature of the experiment The experiments were performed on a Krogh bicycle ergometer at 60 pedal revs/min Work loads were chosen so that each subject would reach a heart rate of about 170 beats/min after 5 minutes work (see Table I) The subjects came to the laboratory in the morning after a light carbohydrate meal They warmed up about 10 min on the bicycle at a lighter work intensity When the pulse rate had returned to near resting levels the subject was told to take the mouth piece and nose clip and from then on the breathed warm and humified gas mixtures delivered to Douglas bags from premixed tanks At this point the subject was breathing either air or 35 % O_2 in nitrogen and after 1—2 min he was told to start pedalling After 6 minutes of work two Douglas bags were collected for $\dot{V}_{O_2}$ determinations and blood samples were drawn from the finger tip of a hand prewarmed with an electric heating pad Then the expiratory air was led into a 150 l balanced Tissot spirometer fitted with a potentiometer the output of which was fed into a recorder (Visicorder model 1706 yielding a staircase function of volume displacement over time from which the breath to breath analyses were made

After about 30 l had passed into the spirometer the inspired mixture was abruptly changed (on expiration) without the subject's awareness to 35 % or 100 % O_2 and a mark placed on the recorder tracing All efforts were made to keep the gas temperatures the same and a drape intercepted the subject's view to the stopcock The dead space between the respiratory valve and the stopcock was 200 ml The spirometer was allowed to fill and the transient recorded Initially when blood samples were to be taken they were performed immediately following the switch After preliminary results showed that the time course of the blood changes did not warrant these immediate blood samples the sampling was postponed until the Douglas bag collection following the spirometer tracing so as not to interfere with the breath to breath analysis When the spirometer was full the expired air was led into a second Douglas bag while the spirometer was emptied Thereafter the expiratory air was again directed to the spirometer and if another switch was to be made the procedure was repeated When the spirometer was full a third Douglas bag was collected while the spirometer was emptied in preparation for the final switch back to air or 35 % O_2 The total time for the experiment was about 20 min and no subject performed more than once per day and on only one occasion more than 3 times per week

Gas samples from the Douglas bags were collected in mercury filled sampling tubes and analysed by micro-Scholander technique Blood P_{CO_2} and pH were measured using Radiometer micro electrodes (type E 5036 and E 5021) and the blood lactate by an enzymatic method (Biochemia Test Combination TC-B 15972, Boehringer und Soehne GmbH Mannheim)

Respiratory frequency was recorded by means of a strain gauge manometer Heart rate was counted from ECG by a pulse recorder which ran continuously or by palpation

The respiratory transients were determined by measuring the volume displaced per time and were calculated in L/min BTPS for each breath Large variations from breath to breath made analysis difficult Therefore for each transient the 6 breaths the switch were averaged

after expressed as a percentage

ent difference and elapsed time

plotted as a single point The

P_{ACO_2} was calculated with the Bohr formula using an assumed dead space derived from the data of *Asmussen and Nielsen* (1956)

Results

Table I shows the absolute values of air breathing obtained after 6—10 min of exercise Because of the training effect of the severe work it was necessary to change the workload several times in the experimental period in order to keep the relative workload constant for each subject Both the mean heart rates and the mean lactate concentrations of the different subjects show that the relative workload was fairly constant from one subject to another

The results from the blood sampling are expressed in relation to the values in Table I the ventilatory findings both the transient and the bag data are treated as mentioned above

Ventilatory results

After the data for each breath was plotted as a percent of the 6 breaths before the switch (as described in Methods) smooth curves were drawn by eye through these points (*cf* Fig 1) These curves were then grouped together on 4 sets of axes one for each condition (A 100 Fig 2 A 35 Fig 3 35—100 Fig 4 100→A Fig 5) Thus each curve represents the experiments on one subject at a given condition

There was a wide variation from breath to breath for one subject (*cf* Fig 1 where S E is shown for all first breaths second breaths and so on) and from subject to subject for any given condition Though this limits the possibility of mathematical analyses there are several points that become apparent upon examining the data

As confirmed by the Douglas bag data the ventilation is depressed when the subject is switched to a gas mixture with a higher oxygen tension than the one he had been breathing There is a greater depression (10—16 % Fig 2 when 100 % O than when 35 % O is breathed 4—8 % Fig 3)

The onset of the depression occurred generally within the first 5—6 sec (Fig 2—Fig 3—Fig 4 and in some cases already the first breath or two are lower Once started the response is quite fast reaching completion by 20—30 sec after the switch In some cases the ventilation tended to overshoot with a subsequent slower clim

TABLE I

Subject	Workload kpm/min		Heartrate beat/min	$\dot{V}_{E\,BTPS}$ l/min	$\dot{V}_{O_2}$ l/min
NR	1460	mean	172 0	79 2	3 305
		s e	2 0	1 0	0 072
	1537	mean	173 6	87 4	3 410
		s e	0 9	1 61	0 015
	1614	mean	—	88 5	3 490
		s e	—	—	—
	1651	mean	181 7	97 15	3 620
		s e	1 6	1 36	0 011
PB	1460	mean	170 9	92 7	3 606
		s e	1 1	1 23	0 03
	1537	mean	165 3	86 14	3 601
		s e	1 1	2 28	0 031
B	1537	mean	163 5	97 80	3 777
		s e	1 20	2 51	0 028
	1651	mean	159 5	101 50	3 846
		s e	1 09	1 30	0 053
	1729	mean	162 8	109 87	4 066
		s e	0 94	1 10	0 021
J	1075	mean	176 0	68 25	2 642
		s e	—	—	—
	1190	mean	174 5	76 65	2 817
		s e	1 66	2 21	0 060
	1229	mean	170	80 06	2 98[illegible]
		s e			—
	1306	mean	173 5	83 32	3 103
		s e			
	1345	mean	169 0	80 55	2 9[illegible]
		s e			

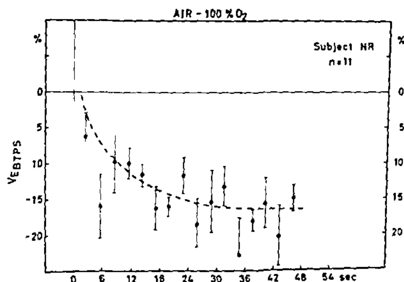


Fig 1 Every single dot represents the mean of 11 experiments first breath second breath third breath and so on The curve is a mean curve drawn by eye ±1 S E is marked around every dot

R	f frequency min	V_T liter	P_{ACO_2} mmHg	pH	Pa_{CO_2} mmHg	Lactate mg %
0 91	22 0	3 465	36 9	7 341	33 1	48 5
0 012	0 32	0 053	0 36	0 003	1 0	1 6
0 93	24 7	3 640	35 3	7 309	34 5	67 0
0 01	0 38	0 080	0 24	0 005	1 0	8 1
0 94	26 0	3 404	35 8	—	—	67 0
—	—	—	—	—	—	—
0 97	25 0	3 895	34 2	7 303	—	71 2
0 01	0 59	0 057	0 35	0 023	—	4 5
0 87	28 8	3 046	35 0	7 333	34 8	50 5
0 03	0 45	0 053	0 34	0 032	0 7	1 6
—	—	—	—	—	—	—
—	—	—	—	—	—	—
1 00	33 0	2 980	37 1	7 285	—	51 5
0 01	1 5	0 098	0 22	—	—	—
0 97	30 0	3 425	35 9	—	—	55 0
0 01	0 0	0 57	0 82	—	—	—
0 97	31 6	3 485	34 8	—	—	—
0 007	0 55	0 060	0 30	—	—	—
0 97	29 0	2 359	37 9	7 340	—	51 5
—	—	—	—	—	—	—
0 98	30 8	2 491	35 7	7 313	—	59 7
0 018	0 37	0 089	0 30	0 041	—	2 0
0 99	32 0	2 620	36 1	7 330	—	49 5
—	—	—	—	—	—	—
0 99	33 0	2 526	36 4	—	—	49 5
—	—	—	—	—	—	—
0 99	34 0	2 370	35 9	—	—	—
—	—	—	—	—	—	—

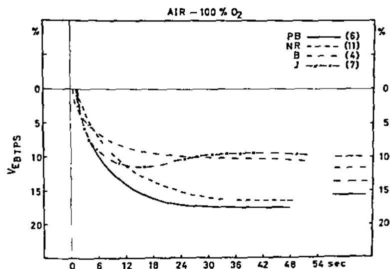


Fig 2 Mean curves from the transients from air 100 % O_2 Horizontal signatures outside the time scale show the depression measured from the Douglas bags (see text)

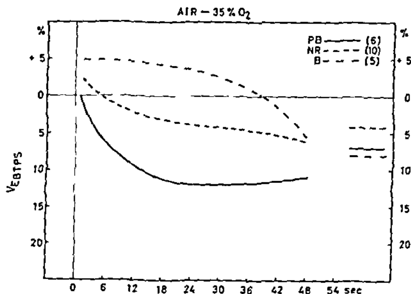


Fig 3 Transients from air 35 % O_2 See also text to Fig 2

back up again (Compare the Douglas bag values with the transient depressions Fig 2—3—4)

Given the wide scattering of the points it is difficult to differentiate between the three conditions of depression except for the magnitude of the depression However the A 35 and 35—100 transients are quite fast and probably have a time course similar to that of the A 100 % transient

Fig 5 shows the off response (100 A) The ventilation begins immediately to increase towards pre switch values Since the baseline from which the percentage differences are calculated (the 6 last breaths during oxygen breathing) are lower than during switch from A 100 the percentage increase of the ventilation after oxygen breathing becomes higher than the percentage depression on the same condition But even using the same baseline in the calculation the percentage increase is still some few percents higher than the depression seen in the same experiment The reason is that the subjects at these high workloads are not in a real steady state but throughout the experiment have a rising ventilation The time course of the off response is much longer than the time course for the depression of ventilation Fig 5 shows 30 to 40 sec

Blood sampling results

Fig 6 shows the change in the blood parameters following the switch The pH is always decreased or unchanged after less than one minute and Pa_{CO_2} is always increased or unchanged after less than one minute

Blood lactate concentrations are changed minimally after one minute With but one exception the tendency is towards a slight increase

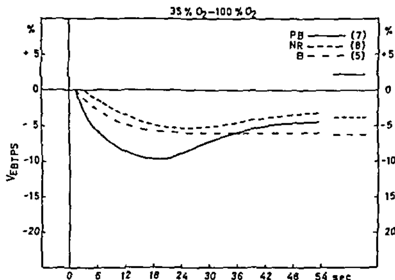


Fig 4 Transients from 35 % O —100 % O2 See also text to Fig 2

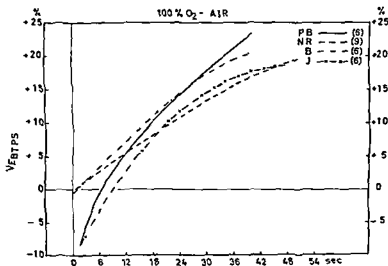


Fig 5 Transients from 100 % O2 air

The change in heart rate was for all 4 subjects minimal and not significant though in air 100 % O there was a slight tendency for the heart rate to decrease by some few beats/min Because of the small number of experimental data in the off response period these are not included in Fig 6 It is obvious that the tendency is the opposite of what occurs during the on response *pH* is increasing or unchanged *after 1 min* P_{CO_2} decreasing or unchanged after 1 min and blood lactate concentration unchanged after the first minute

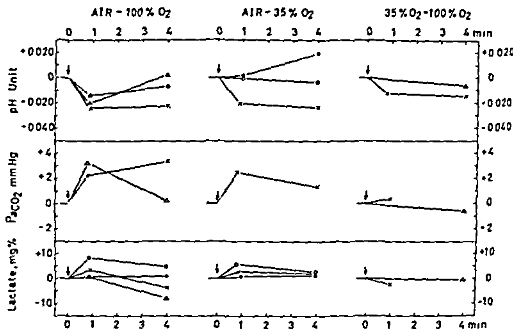


Fig 6 The change in pH Pa_{CO_2} and lactate concentrations found 0 8 min after switch and 4 min after switch Signatures

subject WR x — x subject PB △—△ subject J ○—○ and subject B ●—●

Discussion

The aim of the study was to determine whether the depression of ventilation produced by breathing hyperoxic mixtures during heavy exercise was the result of some fast nervous reflex (*e g* the chemoreceptor drive from the aortic and carotid bodies Bannister and Cunningham (1954) or of a slower metabolic effect (*e g* elimination of some anaerobically produced work substance Asmussen and Nielsen (1946)) or *some combination of the two Thus we first had to define the time course of the fall* in ventilation and then compare this to the rate direction and magnitude of any changes occurring in the pH P_{CO_2} and lactate concentration during this period

As seen in Fig 2 (A 100) the depression starts within the first five seconds after the switch and from the actual breath to breath data it was apparent that the first breath is already disturbed In most cases this is seen as a depression but in some instances the ventilation was significantly elevated on the first breath This immediate effect of hyperoxia seems to be in agreement with the results of Dripps and Comroe (1947) Dejours (1957) Perret (1959) Brady *et al* (1963) and Downes and Lambertsen (*1966*

The full depression was reached after 10—12 breaths or 20—22 sec The entire time course agrees quite well with Lambertsen s data obtained during hyperventilation at rest when the ventilation was driven by CO This agreement suggests that the initial response was independent of the velocity of the blood flow and the size of the cardiac output Support for this independence is also provided by the similarity

in the time courses for the four subjects who were working at widely different work intensities (though at a constant relative workload) The similar time course in exercise and rest suggest that a specific 'work factor' has no role in the immediate response to oxygen breathing

Fig 3 (A-35) shows a wide variation in rate of response from subject to subject Subjects NR and PB reacted with a time course similar to the A-100 (Fig 2) and 35—100 (Fig 4) experiments although PB who was the most sensitive in all conditions had a faster and greater reaction The reason for subjects B s diverging response remains unexplained

In Fig 4 (35—100) it is apparent that the ventilation follows a path similar to the A 100 (Fig 2) condition reaching completion in about 20 sec It is remarkable that A 100 and 35—100 produce ventilatory changes with the same time course if one expects that 35 % oxygen suffices to silence the chemoreceptors If this further depression had been caused by some metabolic factor one would expect some delay and/or a longer time course We can think of two possible explanations for this observation One is that 35 % did not produce a high enough tension to silence the chemoreceptors, possibly because exercise raises the threshold of the chemoreceptors (as suggested by Purves and Biscoe (1968)) The other possibility is that the chemoreceptor mechanism in the aortic and carotid bodies is not responsible for this initial phase The fast response time and the probable independence of the size of the cardiac output and velocity of blood flow would then indicate that some local mechanism in the airways or lungs might be responsible

With the time course of the ventilation well defined one can now examine the changes in blood lactate, pH and Pa_{CO_2} (Fig 6) to determine their possible role in the ventilatory changes In the first blood sample after the switch pH and Pa_{CO_2} had already changed from control values However the direction of these changes is opposite from what one would expect were they causative factors Probably they are results and not causes of the altered ventilation Lactates on the other hand had not changed significantly in the first blood sample (if any change it went up) suggesting that it was not responsible for the decline in ventilation

In the following blood samples the mean pH had risen slightly and the mean Pa_{CO_2} decreased slightly but still pH is lower and Pa_{CO_2} higher than the control values, while the lactate had begun to fall The results agree with those of Asmussen and Nielsen (1946) Bannister and Cunningham (1954) and Perret (1960) While the pH and P_{CO_2} are clearly results of the ventilatory changes the lowered lactate may reflect a decreased anerobicity in the working muscles

The magnitude of the ventilatory depressions produced by 100 % O as measured by both the breath to breath method and the Douglas bag method was of the order of 10—16 % This agrees well with the result that Asmussen (1950) and Bannister and Cunningham (1954) (66 % and 100 %) and Perret (1960) (60 %) found during exercise while Asmussen and Nielsen (1946 1958) found a somewhat higher depression of 17—20 % Downes and Lambertsen s, Dejours and Brady s results in rest gave the same percentual decrease in ventilation as found during exercise

It is interesting that the initial response to oxygen breathing represents the same proportion of the ventilation in both rest and exercise experiments on adults in new borns and in studies where the ventilation was driven by CO In Brady s experiments one would expect as Downes and Lambertsen point out that the newborn with a large A a PO gradient would have a larger chemoreceptor component and hence a larger per cent reduction But as mentioned before it is possible that the chemoreceptors are not responsible for this initial depression

As seen from Fig 3 the depression from air to 35 % O is only 4—8 % judged from the bag level This finding agrees with Asmussen and Nielsen s and Bannister and Cunningham s findings The change from 35—100 % (Fig 4) shows a depression of 4—7 % but since 35 % O has been breathed from the beginning of the experiment the startpoint (100 % ventilation) is around 4—8 % lower than when air is breathed If this is corrected the total depression is about the same as in the air 100 % experiments

Although there is a slight tendency for the ventilation to rise during the oxygen breathing period the ventilation still maintains its depression throughout the oxygen breathing period This is contrasted by Downes and Lambertsen s experiments during rest (V_F driven by CO) in which the ventilation rose to normal after about 50 seconds of oxygen breathing Also Dejours results in rest show the same trend The present findings suggest that something connected with heavy exercise is responsible for maintaining the depression in ventilation beyond the first minute It is possible that the alleviation of local anerobicity in the working muscles during heavy exercise is equivalent to a decrease in the sum total of stimuli acting on the respiratory center in heavy exercise This explanation is corroborated by the work of Perret who found that at low and moderate work loads 40 % O would produce an initial 10 % depression of ventilation which again would rise in 2—3 min to near control levels In heavy exercise however this initial response was followed by a sustained depression Further evidence of this metabolic effect of O is data of Asmussen and Nielsen (1946 1958) Asmussen (1950) and Bannister and Cunningham (1954) and the present experiments that show a difference in the steady state ventilation between 30—35 % and 60—100 %

The off response (Fig 5 100 A) shows that the time course for ventilation is longer than the observed time course seen on the on response (Fig 2—3—4) The experiments of Leitner *et al* show the same trend on the depression and rise of ventilation following two breaths of pure oxygen. Furthermore their study shows that the changes in ventilation follow the discharge frequency of the chemoreceptors This agreement therefore seems to indicate that the chemoreceptor activity at least in the transition period plays a very important role The different reaction time for the chemoreceptors at on response and off response can not be caused by differences in oxygen tensions in the blood From our data we have calculated that the change in oxygen tensions are complete after 10—12 seconds both on on response and off response The different time course may therefore be connected to the behavior of the chemoreceptors themselves

It must be concluded that the initial depression to oxygen breathing during severe exercise probably is caused by the same factors as in rest since the time course of ventilation is the same in rest and exercise If the aortic and carotid chemoreceptors are responsible for the depression in ventilation it may be concluded that the threshold values of these organs are changed during severe exercise so that 35 % O is not enough to silence them If the chemoreceptors are not involved in this initial depression of ventilation the effect may be connected to some local mechanism in the lungs of airways, as already the first breath is disturbed Furthermore the similarity of the time course from air to 100 % and from 35 % to 100 % O and the immediate response to changes indicate that the response must be of the same nervous origin The changes in pH, Pa_{CO_2} or lactate can not be the causative factors as the changes in these parameters are contrary to what should be expected if they were responsible for the change The maintained depression in ventilation in severe exercise caused by oxygen breathing in contrast to results from rest experiments may be explained by a decrease in anerobicity in the working muscles as we found a decreasing tendency in the blood lactate concentrations If this is true it supports Stegemann's (1965) postulation of respiratory chemoreceptors located in the muscles

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The Distribution of 15-Hydroxy Prostaglandin Dehydrogenase and Prostaglandin-Δ^{13}-Reductase in Tissues of the Swine

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Abstract

ÄNGGÅRD E C LARSSON and B SAMUELSSON *The distribution of 15 hydroxy prostaglandin dehydrogenase and prostaglandin-Δ^{13}-reductase in tissues of the swine* Acta physiol scand *1971 81* 396—404

Prostaglandin dehydrogenase (15-hydroxy prostanoate oxidoreductase EC 1 1 1 (PGDH) and prostaglandin Δ^{13} reductase (prostanoate Δ^{13} oxidoreductase) were assayed in homogenates of 15 different tissues of the swine Both enzymes were located mainly in the particle free fraction The highest activities for PGDH were found in the lung spleen and kidney which ranged from 50—82 mU/g of tissue or from 482 to 808 mU/g of protein Lower activities of PGDH were observed in the stomach testicle liver and small intestine The renal cortex contained about three times more PGDH than the medulla The reductase was most abundant in spleen kidney liver adrenal and small intestine which ranged between 27—38 mU/g of tissue or 232—390 mU/g of protein The highest specific activity was however found in adipose tissue which contained 3383 mU/g of protein The wide distribution of these enzymes as well as other evidence support the concept that they catalyze the initial reactions in the metbolism of the prostaglandins

In the lung prostaglandin E compounds[1] are metabolized by oxidation of the secondary alcohol group at carbon atom 15 to a ketone and also by reduction of the Δ^{13} double bond (Änggård and Samuelsson 1964 Änggård Green and Samuelsson 1965, Änggård and Samuelsson 1965) In swine lung only the former reaction has been demonstrated (Änggård and Samuelsson 1966) The enzyme responsible for this conversion 15 hydroxy prostaglandin dehydrogenase (EC 1 1 1) was extensively purified from swine lung (Änggård and Samuelsson 1966) and shown to be specific for the C 15 (S) alcohol group in the prostaglandins (Änggård and Samuelsson 1966, Nakano Änggård and Samuelsson 1969 Shio *et al* 1969) It seems likely that the dehydrogenation represents a biological inactivation of the prostaglandins since the 15 keto metabolites were shown to be considerably less active in several biological systems (Änggård 1966 Pike, Kupiecki and Weeks 1967) The importance of

[1] For a review see Bergstrom Carlson and Weeks (1968)

this pathway is further born out by the fact that the major urinary metabolit f prostaglandin E_1 (PGE_1), prostaglandin E_2 (PGE_2), prostaglandin $F_{1\alpha}$ PG and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in guinea pig rat and man were shown to hav n 15 keto structure (Granström and Samuelsson 1969 a, b Green 1969 Hamberg a d Samuelsson 1969 a, b)

The prostaglandin Δ^{13} reductase has not yet been purified for studies of its properties Evidence for its presence has been demonstrated in guinea pig rat and man, since metabolites of the PGE and PGF-compounds lack the double bond in these species (Ångård and Samuelsson 1964, 1965, Änggård Green and Samuelsson 1965, Granstrom and Samuelsson 1969 a, b Green 1969, Hamberg and Samuelsson 1969 a, b) Studies on the biological activity of dihydro-PGE_1 showed that this compound was less active than PGE_1 on several isolated smooth muscle preparations but about equally active on the rabbit guinea pig and rat blood pressure (Ångård 1966, Pike, Kupiecki and Weeks 1967)

Since both the reactions mentioned above seem to represent biologically important reactions in the catabolism of the prostaglandins their distribution in different organs is of interest. The present paper gives quantitative data on the occurrence of the 15 hydroxy prostaglandin dehydrogenase and prostaglandin Δ^{13} reductase in tissues of the swine

Materials and methods

Organs and tissues from either castrated female or male adult swine were collected at slaughter frozen on dry ice and stored at $-20°$ C The intestines were rinsed in cold water Adipose tissue was taken from the fat pad on the abdominal side of the dorsal root of the diafragm The muscle was taken from the diaphragm Usually tissues from several animals were pooled and used for repeated analyses Assays were conducted within four weeks of storage No loss of act

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NADH for various times It has been found (Änggard and Larsson unpublished information that NADH stimulates the rate of reduction of 15 keto-PGE_1 in the supernatant fraction of swine kidney homogenates

The frozen tissues were weighed cut into smaller pieces and allowed to thaw in cold 0 1 M potassium phosphate buffer pH 8 0 containing 0 06 % mercaptoethanol The tissue to buffer ratio was adjusted to 1 4 (weight/vol) and homogenized twice for about 2 min at 0° C using

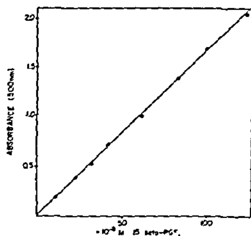


Fig 1

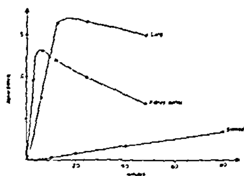


Fig 2

Fig 1 Linearity between 500 nm absorption and concentration of 15 keto-PGE₁ after addition of alkali The 15 keto-PGE₁ was dissolved in 2 ml of 96 % ethanol After addition of 0 2 ml of 1 N NaOH the absorption at 500 nm was read when it had attained the maximum value

Fig 2 Time course for the appearance and disappearance of 15 keto-PGE₁ formed from PGE₁ in extracts from lung renal cortex and stomach For details see Experimental

for NAD or NADH Incubation at 37° C with shaking was started immediately after the addition of the substrates The enzymatic reaction was stopped by the addition of 25 ml of ice cold 99 % ethanol The proteins were left to precipitate overnight at room temperature

After filtration and washing of the precipitate with 5 ml of ethanol the combined filtrates were evaporated until dryness *in vacuo* in a round flask The dry residue was taken up in 2 ml of water and 2 ml of petroleum ether (b p 30—60°) This mixture was transferred to a 10 ml centrifuge tube After stirring the organic layer was removed and discarded The round bottomed flask was further rinsed twice by a small volume of ethyl acetate which previously had been equilibrated with 0 15 N HCl These acid ethyl acetate rinses were added to the aqueous phase in the test tube After acidification with 1 drop of 6 N HCl extraction was performed twice with about 2 ml of ethyl acetate The combined organic phases were washed twice with 0 5 ml of H_2O Brief low speed centrifugation was used to aid separation of the phases The ethyl acetate was evaporated to dryness

The extract was dissolved in 2 ml of 96 % ethanol The amount of 15 keto-PGE₁ was measured at 500 nm in a Zeiss model PMQ II spectrophotometer after addition of 0 2 ml 1 N NaOH Under these conditions the absorption rose to a peak value in about one minute was stable for about another minute and then gradually declined over the next hour The absorption is linear from 10⁻⁷ to 10⁻⁴ M of 15 keto-PGE₁ (Fig 1) The apparent molecular extinction coefficient under these conditions was 16 900

The recovery of prostaglandins through the extraction procedure was studied using ³H labelled PGE₁ and found to be 80 5 ± 5 2 % (Mean ± SD n 7) The protein was determined according to Lowry *et al* 1951

Subcellular distribution of prostaglandin metabolizing enzymes

[illegible] homogenate was prepared and subjected to centrifugation as described above The [illegible]

and the supernatant fractions were adjusted to [illegible] homogenate Assays for PGDH and Δ¹³ reductase were carried out as described above

TABLE I The subcellular distribution of 15-hydroxy prostaglandin dehydrogenase and prostaglandin-Δ^{13} reductase The mean and standard deviation from three determinations are given

Tissue	PGDH mU/g tissue		Δ^{13} reductase, mU g tissue	
	Supernatants	Particles	Supernatants	Particles
Kidney	112 3±36 9	28 0±93	39 6± 6 5	0 88±0 8
Spleen	93 5± 9 9	23 4±5 4	34 5±15 4	6 55±5 3
Lung	77 3± 5 2	23 1±2 3	22 0± 8 9	0 75±0 9

Results

Time course of the 15-hydroxy prostaglandin dehydrogenase (PGDH) reaction

Preliminary studies showed that in the supernatant fraction from kidney and lung the time course for the formation of 15-keto PGE_1 from PGE_1 had biphasic appearance This is exemplified in Fig 2 After an initial rise in the concentration of the product there was a linear decline By isolation and structure determination of the products formed from 15 keto-PGE_1 it could be established that the decline in 500 nm absorption was due to the action of the Δ^{13}-reductase (Änggård and Larsson, 1971 Thus the increase in 15-keto-PGE_1 following incubation with PGE_1 represents the PGDH activity which however must be corrected for the simultaneous rate of reduction of 15-keto-PGE_1 The activity of the Δ^{13} reductase was determined in different experiments using 15-keto-PGE_1 as substrate in the presence of NADH

The subcellular distribution of 15 hydroxy prostaglandin dehydrogenase and prostaglandin-Δ^{13} reductase

The amounts of these enzymes were studied in the 100 000 g supernatant and the corresponding particle fraction of kidney spleen and lung The results are shown in Table I It is seen that most of the activity of the PGDH and the Δ^{13}-reductase was present in the supernatant fraction

Distribution of 15-hydroxy prostaglandin dehydrogenase

The amount of PGDH present in the supernatant prepared from a number of tissues from the swine is shown in Fig 3 These are calculated from the rate of the oxidation of PGE_1 and are correcter for the simultaneous reduction of 15-keto PGE_1 The highest activities were found in the lung spleen and kidney where 50 3—82 4 mμmoles were oxidized/min/g of tissue Lower rates of oxidation were noticed in stomach testicle small intestine and liver Low but significant activities were found in brain uterus ovary adipose tissue heart muscle adrenal and pancreas About three times higher activity was found in renal cortex as compared to medulla

TABLE II Specific activity of 15-hydroxy prostaglandin dehydrogenase and prostaglandin-Δ^{13}-reductase in the supernatant fractions from homogenates of various organs from the swine Each value is the mean (±SE) from 3 exp

Tissue	PGDH activity mU/g of protein	Δ^{13}-reductase activity mU/g of protein
Kidney	808 ± 220	248 ± 79
Spleen	651 ± 0	232 ± 37
Lung	482 ± 73	197 ± 44
Liver	115 ± 19	173 ± 14
Intestine	117 ± 46	390 ± 140
Stomach	76 1 ± 15	133 ± 91
Adrenal	56 7 ± 19	322 ± 150
Pancreas	33 6 ± 4 7	157 ± 17
Heart	38 9 ± 13	67 6 ± 56
Adipose tissue	701 ± 120	3380 ± 890
Muscle	21 9 ± 2 2	19 0 ± 17
Testicle	111 ± 10	230 ± 90
Uterus	24 9 ± 2 1	111 ± 24
Ovary	29 9 ± 3 4	217 ± 2 1
Brain	65 1 ± 2 3	468 ± 110

A somewhat different pattern is seen if the specific activity (mμmoles/min/g of protein) is calculated for the different organs (Table II) The absolute amount of PGDH in adipose tissue was fairly low (Fig 3) However, the specific activity was higher than the activity of the lung (Table II) an organ with known high levels of PGDH (Ånggård and Samuelsson 1966)

In 3 expts the PGDH activity from lung, spleen, kidney, liver, stomach, intestine and heart, was compared in tissues from one boar, one sow and from a pool of tissues obtained from castrated animals No marked difference in the levels of PGDH was observed

Distribution of prostaglandin-Δ^{13}-reductase

The distribution of Δ^{13}-reductase activity is shown in Fig 4 The highest amounts were found in the spleen kidney, liver, adrenal and intestine having 26 6—37 9 mμmoles/min/g of tissue The activity in the other organs ranged between 2 0—25 8 mμmole/min/g of tissue

The specific activity is shown in Table II Adipose tissue had the highest specific activity followed by kidney adrenal, intestine and brain

Discussion

The enzymes responsible for the oxidation of the secondary alcohol group at carbon atom 15 and for the reduction of the Δ^{13}-double bond in the prostaglandins were first demonstrated in homogenates of lung tissue of guinea pig (Ånggård and Samuelsson 1964, Ånggård *et al* 1965 Ånggård and Samuelsson 1965) Preliminary experiments showed that these reactions occurred also in other tissues such as

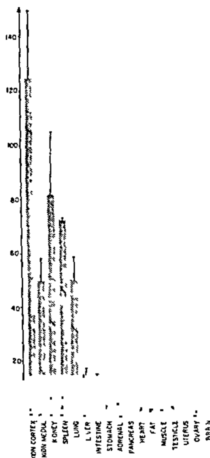


Fig 3

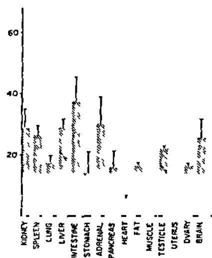


Fig 4

Fig 3 The activity of 15 hydroxy prostaglandin dehydrogenase (PGDH) in the supernatant fractions from homogenate of various organs from the swine Each value is the mean (±SE) from three experiments Essentially similar results were obtained in a number of preliminary experiments using slightly different incubation conditions

Fig 4 Activity of prostaglandin Δ^{13} reductase in the supernatant fractions from homogenates of various organs from the swine Each value is the mean (±SE) from 3 expts

intestine and kidney (Änggård and Samuelsson 1966 b) Recently histochemical evidence for the presence of PGDH in the rat kidney has been reported (Nissen and Andersson 1968 1969) The present investigation was undertaken as a more systematic study of the occurrence of these enzymes in the swine

Our results show that by far the highest levels of PGDH are seen in the kidney, spleen and lung Biosynthesis and/or release of prostaglandins have been demonstrated in all these organs (Änggård and Samuelsson 1965 b, Lee *et al* 1967, D

et al 1968, Edwards *et al* 1968, Gilmore *et al* 1968, Crowshaw, 1969, Davis 1969, Fujimoto and Lockett 1969, Hamberg 1969) In fact, next to the sheep vesicular gland, the lung, spleen and kidney are the tissues where the highest rates of biosynthesis and/or release have been observed Our finding that these organs also contain high levels of prostaglandin dehydrogenase indicates a high turnover of prostaglandins in these organs

The levels of prostaglandin dehydrogenase in renal cortex were 2—3 times higher than those found in the medulla This would agree fairly well with the histochemical data of Nissen and Andersen (1968) These investigators found that in the rat the most pronounced activity was observed in the thick ascending limb of the loop of *Henle which would be situated mostly in the cortex and outer medulla In this con*nection it might be remarked that the occurrence and biosynthesis of renal prostaglandins have been demonstrated only in renal medulla (Lee *et al* 1967, Crowshaw 1969 a, b, Hamberg 1969) It is possible that the high prostaglandin dehydrogenase activity in the cortex (Fig 2, 3) rapidly inactivates the prostaglandins there which thus escape identification

Our results clearly do not agree with those of Crowshaw (1969 b) who in the rabbit kidney found no evidence for cortical mechanisms of inactivation of prostaglandin E_1 Species variations may account for these differences

The activity of PGR was about the same as PGDH in the organs except kidney, spleen and lung, where the PGDH activity was much higher In the organs with high dehydrogenase activity like kidney and spleen the reductase activity was also fairly high The lung had, as previously observed (Anggård and Samuelsson 1966 a), a fairly high ratio between the levels of dehydrogenase and reductase In the liver, small intestine, adrenal and adipose tissue the PGR activity was slightly higher

Adipose tissue deserves mentioning in this context because it had the highest specific activity of Δ^{13}-reductase and also fairly high dehydrogenase activity This evidence for prostaglandin metabolism in the fat is of interest since prostaglandins have been shown to be formed in this tissue (Shaw and Ramwell 1968) and are potent inhibitors of noradrenaline induced lipolysis (Steinberg *et al* 1963, 1964, Bergstrom *et al* 1964 Bergstrom *et al* 1968)

Studies on the metabolism of prostaglandins in the liver has revealed that these compounds undergo β oxidation in one or two steps giving rise to dinor and tetranor metabolites (*Hamberg 1968*) *Evidence for β oxidation has also been found* in the intestine (Parkinson and Schneider 1969) The major urinary metabolites of the PGE and PGF-compounds have β oxidized carboxylic side chains and have in addition the saturated 15-keto structure (Granstrom and Samuelsson 1969 a, b Green 1969, Hamberg and Samuelsson 1969 a b) As shown by the present experiments the prostaglandin dehydrogenase and reductase have a wide tissue distribution This finding and recent experiments demonstrating that di and tetranor prostaglandin E_1 and F_1 are poor substrates for prostaglandin dehydrogenase (Nakano *et al* 1969) indicate that the catabolism of the prostaglandins is initiated by the reactions catalyzed by the 15 hydroxy dehydrogenase followed by the Δ^{13}-reductase reaction

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Effects of Variations in the Extracellular Osmolality on the Ionic Permeability of Vascular Smooth Muscle

By

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Abstract

JONSSON, O, *Effects of variations in the extracellular osmolality on the ionic permeability of vascular smooth muscle* Acta physiol scand 1971 *81* 405—421

The effect of osmolality variations on the membrane permeability of vascular smooth muscle to sodium and potassium was investigated b
cellular tonicities The K efflux in norm
M/cm² sec at 15° C The corresponding
assuming a membrane potential of 50 mV

Previous studies concerning the effects of varied osmolality on the electrical and mechanical activity of the rat portal vein revealed a correlation between volume and degree of activity of the muscle cells Osmotic manipulations that make the muscle cells swell cause excitation and procedures that make them shrink cause inhibition of their spontaneous activity (Mellander et al 1967, Johansson and Jonsson 1968 Arvill Johansson and Jonsson 1969 Jonsson 1969 a, b) Simultaneous variations in ionic composition and osmolality of the incubation medium indicated that the link between these alterations in cell volume and activity could not consist only of the concomitant changes in transmembrane ionic concentration gradients It was therefore suggested that shrinkage or swelling of the cells may also influence the permeability properties of the cell membranes For example, a relatively stronger influence on the permeability constant for sodium than on that for potassium, as a

cell volume variations, would potentiate the effects of alterations in the ionic concentration gradients and would thus contribute to the changes in activity (Mellander *et al* 1967, Johansson and Jonsson 1968, Jonsson 1969 a)

The purpose of the present investigation was to explore this possibility by studying the transmembrane effluxes of ^{24}Na and ^{42}K in the portal vein at different extracellular osmolalities, which might give evidence for such osmotically induced changes in the permeability constants for sodium and potassium In a concomitant study (Jonsson 1971), some *general* aspects of membrane permeability in the vascular smooth muscle were elucidated by examining the exchange kinetics of two non electrolytes, urea and erythritol, under different environmental conditions

Methods

[illegible]

thawed again preincubated and treated exactly like the fresh preparations used for measurements of Na and K fluxes

Uptake of ^{24}Na and ^{14}C sucrose

After the preincubation period the preparations intended for ^{24}Na uptake were allowed to adapt to 15 C for another 30 min period When the uptake was studied in an anisosmotic solution they were exposed to the composition of this medium for an additional 15 min The preparations were then loaded for either 10 or 30 min at 15 C with ^{24}Na (initial specific activity in the medium approximately 0 05 mc/mmole Na) in one of the following three solutions 1) normal Krebs 2) Krebs plus 150 mmoles sucrose/l and 3) Krebs minus 30 mmoles NaCl/l After the incubations the muscles were gently blotted between two pieces of filter paper according to a standardized time schedule weighed on a Cahn electrobalance and homogenized in 10 % tri chloro acetic acid The radioactivity of the muscle extracts and media was determined with a Packard Tri Carb liquid scintillation counter (for details see Arvill *et al* 1969)

The extracellular space under the abovementioned experimental conditions was determined by measuring the 15 min uptake of ^{14}C sucrose (5 0 mc/mmole 0 25 mmole/l), exposing the portal vein preparations to exactly the same incubation conditions as those used in the ^{24}Na uptake experiments

Measurements of potassium and sodium fluxes

After preincubation the portal veins intended for studies of the Na or K effluxes were exposed for 2 hrs to either ^{24}Na or ^{42}K (initial specific activity in the medium 1 and 0 7 mc/mmole respectively) Earlier uptake experiments had shown that the used period of incubation was sufficient for ^{42}K to equilibrate with the total exchangeable fraction of potassium (Haljamäe *et al* 1970) For those preparations where the subsequent washout was performed either at 15° C or in anisosmotic solutions instead of in normal Krebs at 37° C the last 30 and 15 min respectively of the 2 hrs loading period were accomplished in the same temperature and osmotic environment as utilized ... through

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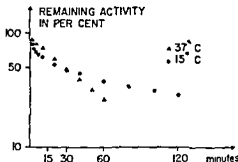


Fig 1 Efflux of ^{42}K from rat portal vein in normal Krebs solution at 37 and 15° C, respectively

284±0 (mean±SE) (n = 36), Krebs plus 150 mmoles sucrose/l 444±1 (n = 16) and Krebs minus 30 mmoles NaCl/l 230±1 (n = 14)

The degree of quenching was estimated by comparing the cpm's obtained at two different pulse height intervals and found to be approximately the same for extracts, incubation media and washout media for one and the same tracer substance

Results

Experiments, where the washout of ^{42}K and ^{24}Na was studied under comparable conditions, were performed in order to investigate whether alterations in extracellular osmolality would influence the permeability coefficient for sodium relative to that for potassium, as outlined above

1 *Potassium washout:* Preliminary experiments showed that, at 37° C, the ^{42}K washout curve, after an initial, more rapid phase, straightened out to a single exponential within a few minutes, as illustrated by the semi-log plot in Fig 1 (triangles) Its rate constant was 0 0180 min^{-1} and the intercept with the ordinate corresponded to a potassium content of 40 6 meq/kg wet weight It is reasonable to assume that this part of the curve represented exchange of *intracellular* potassium, first because the amount of potassium that exchanged with this rate constant gave a figure for $[K^+]_i$ of 137 meq/l, a figure that corresponds closely to that calculated from uptake experiments (Haljamae *et al* 1970), second because this rate constant was of the same magnitude as that reported for cellular exchange of K in other types of smooth muscle (Goodford and Hermansen 1961, Freeman—Narrod and Goodford 1962, Born and Bulbring 1955, Hagemeijer, Rorive and Schoffeniels 1965 b, Goodford 1966, Casteels 1969)

The washout of ^{24}Na in the portal vein turned out to be so rapid at 37° C that it was considered necessary to perform the sodium experiments at lower degrees of temperature In order to obtain comparable conditions, it was thus also essential to study the ^{42}K washout at lowered temperature The filled circles in Fig 1 illustrate such a ^{42}K washout curve, obtained at 15° C in normal Krebs solution Contrary to what was observed at 37° C the curve did not straighten out to become strictly monoexponential until after 60 min The mean rate constant (±SE) for K efflux culated from this late linear part of seven curves, obtained at 15° C in normal solution, was 0 00453±0

exchange of sodium at 15° C was deduced from the interval 7—30 min of the ^{24}Na washout curve (see below) it was considered appropriate to use the same interval for ^{42}K. Such a procedure would imply that the transmembrane passage of potassium and sodium was studied under quite comparable conditions because of identical times of exposure to lowered temperature and to anisosmotic solutions. It should be noticed that, in most cases, the ^{42}K washout *after all occurred at an almost constant rate* also during this earlier phase (correlation coefficient less than —0.996). If this part of the curve (see Fig. 1) was taken to represent efflux of cellular potassium the mean value ($\pm$SE) for the rate constant of its exchange was 0.0126$\pm$0.0008 min^{-1} (n = 7) in normal Krebs solution at 15° C. Corresponding figures for ^{42}K washout in Krebs minus *30 mmoles NaCl/l* were *0.0168$\pm$0.0009 min^{-1}* (n = 7) and in Krebs *plus 150 mmoles sucrose/0.0114$\pm$0.0007* min^{-1} (n = 8). The amount of potassium leaving the muscles with these rate constants was calculated from the intercepts with the ordinate and from the total initial amount of ^{42}K and found to be, in meq/kg wet weight, 25.5$\pm$1.6 (n = 7) for normal Krebs, 24.4$\pm$0.9 (n = 7) for hypotonic Krebs and 33.9$\pm$2.1 (n = 8) for hypertonic Krebs.

2. *Sodium washout.* As mentioned above, the exchange of ^{24}Na turned out to be very rapid at 37° C and therefore the studies on the transmembrane Na fluxes were performed at 15° C. Fig. 2 shows the efflux of ^{24}Na in normal Krebs solution at the latter temperature. The triangles, representing the decrease of radioactivity left in the tissue as a function of time, could after the first 10 min be fitted by a straight line with a $t_{1/2}$ of 14.8 min. When this line was extrapolated to zero time and subtracted from the total curve, a component with a half time of only 1.6 min was revealed. It can also be seen from Fig. 2 that the greatest fraction of the initial amounts of *^{24}Na left the tissue at a still faster rate. The time resolution of the present washout* procedure does not permit a detailed analysis of this early part of the curve.

The amounts of ^{24}Na in the slowest phase corresponded to a sodium content of approximately 4.2 meq/kg wet weight, but this figure could not without further evidence be assumed to represent intracellular sodium. The reason is that a comparatively large fraction of the sodium content in the portal vein appears to be bound in an osmotically inactive form (Haljamae *et al.* 1970). This bound Na fraction was estimated on muscles where the membrane function had been impaired by freezing and thawing, resulting in an apparently homogenous fluid phase equilibrated with the surrounding medium. It was then possible to estimate this bound sodium as corresponding to that fraction which could not be accounted for by sodium ions in the tissue fluid.

The kinetics of the bound sodium fraction is, however, not known and it is therefore not clear whether the sodium washout curve may be treated as a simple two compartment system. It was, however, considered possible that an analysis of the sodium washout from frozen and thawed muscles might help to distinguish between the ionic sodium content in the intracellular fluid and the bound sodium by comparing such washout curves with those obtained with "normal" veins. For such reasons emphasis will be placed on the latter part of the washout curves from the two types

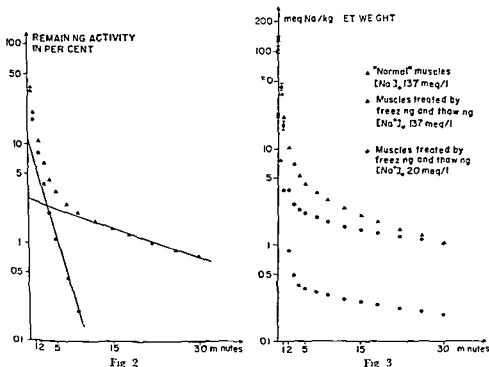


Fig 2

Fig 3

Fig 2 Efflux of ^{24}Na at 15° C The triangles represent the logarithmic decrease of activity as a function of time The linear part of this curve is extrapolated to zero time and subtracted from the total counts giving the values for the faster component (open circles)

Fig 3 Mean values for the efflux of Na at 15° C from 8 normal portal veins washed out in standard Krebs [illegible]

of preparations Fig 3 shows the mean efflux at 15° C of ^{24}Na from normal veins ($n = 8$) in standard Krebs solution (triangles) and from frozen and thawed muscles at two different extracellular sodium concentrations Four of these damaged muscles were kept in an identical medium (open circles) while three were washed out in an isotonic sucrose—substituted medium, where the sodium concentration was reduced to 20 mmoles/l (filled circles) In all three cases the veins had initially been exposed to active ^{24}Na Krebs solution for 90 min at 37° C and then for another 30 min at 15° C before the washout The loss of ^{24}Na from the damaged vessels was after the first 7 min approximately mono exponential and had the same rate constant whether $[Na^+]_o$ was kept at 137 or at 20 meq/l However, the *initial amount* of slow phase sodium turned out to be correlated to the actual $[Na^+]_o$ Figs 4 and 5 illustrate washout of ^{24}Na from normal and damaged muscles in hyper and hypotonic media respectively

The interpretation of these washout curves of damaged muscles and their re[illegible] to those obtained with normal veins is difficult and calls for comments

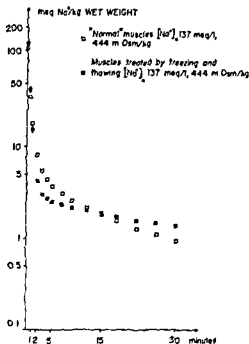


Fig 4

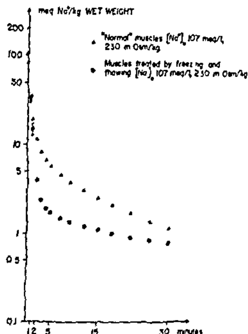


Fig 5

Fig 4 Mean values for the efflux of Na at 15° C from "normal" (open squares) and damaged (filled squares) portal veins in Krebs plus 150 mmoles sucrose/l The standard error of the means has been indicated by vertical bars at points where it exceeded the size of the symbols

Fig 5 Mean values for the efflux of Na at 15° C from "normal" (triangles) and damaged (open circles) portal veins in Krebs minus 30 mmoles NaCl/l The standard error of the means has been indicated by vertical bars at points where it exceeded the size of the symbols

this context The most likely explanation might be that the late, monoexponential part obtained with damaged muscles represents a comparatively firmly bound sodium fraction whose magnitude is dependent on the Na concentration with which the binding sites are equilibrated If the characteristics of these sites were identical in normal and damaged muscles the maximum amounts of sodium that could possibly be bound by intact muscles in normal Krebs would be approximately 2 7 meq/kg wet weight, i e the initial quantity of slow phase sodium equilibrated with an $[Na^+]_o$ of 137 meq/l (open circles, Fig 3) If so, the bound sodium and $[Na^+]_i$ would be washed out in parallel and a subtraction of the curve representing the decline in bound ^{24}Na (open circles) from that representing decline in total ^{24}Na (triangles) should yield a curve for the transmembrane efflux of cellular, ionized sodium

accurately illustrated by the curve obtained in the damaged muscles ... of 20 meq/l An answer to this question is indicated in Fig 4, showing mean values

for the Na washout from "normal" and damaged veins in Krebs solution plus 150 mmoles sucrose/l. The finding, that the curve representing ^{24}Na washout from damaged muscles crossed over that of intact preparations, makes it unlikely that the slow compartment is normally equilibrated with $[Na^+]_o$. It is more likely that it originates from binding sites located *inside* the cells and thus is equilibrated with $[Na^+]_i$ in intact fibres.

On the basis of such reasoning it was considered that a fair estimate of the transmembrane washout would be obtained by subtracting the curve given by *filled* circles from that given by the triangles in Fig. 3. Obviously such a procedure would still be approximate because the exact level of the washout curve for bound sodium is not known and because it would be based on the assumption that the two exponential processes (*i.e.* loss of cellular ^{24}Na, loss of bound ^{24}Na) are coupled in parallel while they in fact are coupled in series (Fig. 4). The first uncertainty could be corrected for by the following method of successive approximations. The $[Na^+]_i$ was estimated from the intercept with the ordinate of the monoexponential part of the Na washout curve obtained with normal muscles (triangles in Fig. 3) and from the volume of cell water per kg wet weight. Since, in the actual range of Na concentrations, the initial amounts of slow phase sodium in damaged muscles was linearly related to the Na concentration in the medium it was possible to find the value for the content of firmly bound sodium that would correspond to the estimated $[Na^+]_i$. A better estimate of $[Na^+]_i$ could then be obtained by subtraction of the bound fraction. This new $[Na^+]_i$ value could be used to recalculate the magnitude of the bound Na fraction and so on. The values finally obtained with this method for calculating $[Na^+]_i$ and firmly bound sodium were 15.7 meq/l cell water and 0.28 meq/kg wet weight, respectively. The latter value corresponds to 1.1 meq/l cell water.

As the rate constant for washout of the bound Na fraction seemed to be independent of the curve level within the range studied (Fig. 3) its efflux should be described by the term $0.28\,e^{-0.131t}$ at a sodium concentration of 15.7 meq/l. The subtraction of this exponential from the [illegible] part of the washout curve obtained with *normal* muscles (triangles in Fig. 3) should reveal the efflux curve of $[Na^+]_i$, if the two processes were coupled in parallel. Now, since the washout of bound ^{24}Na evidently takes place through the intracellular fluid compartment a correction had to be introduced into the calculations. It can be demonstrated that the decrease in bound Na as a function of time under these circumstances proceeds according to the formula

$$Y_t = \frac{Y_0}{\lambda_b - \lambda_i}\left(\lambda_b e^{-\lambda_i t} - \lambda_i e^{-\lambda_b t}\right)$$

which was suggested to the author by Civ. Ing. Lars Stage. Y_0 stands for the initial amount of bound sodium, λ_b and λ_i for the rate constants of the effluxes of bound and intracellular ionized sodium respectively, and t for the time after the transfer to inactive solution. The shape of the washout curve in normal Krebs solution, when this correction for the influence of bound sodium was applied, is illustrated by the filled circles in Fig. 6. After the first 7 min the decrease in activity proceeded alon

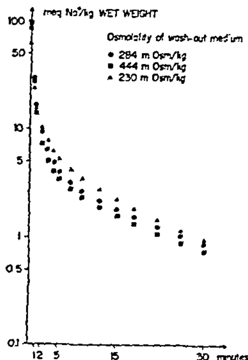


Fig 6

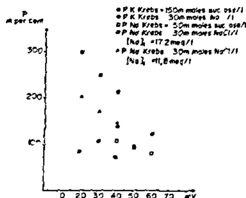


Fig 7

Fig 6 Mean values for the Na efflux from normal portal veins at 15 C at three different extracellular osmolalities after subtraction of the component representing exchange of the small firmly bound sodium fraction The monoexponential parts of these wash out curves have been taken to represent exchange of "free" intracellular sodium

Fig 7 Correlation between the estimated permeability coefficients for K and Na in hyper and hypo-osmotic media and the value for the membrane potential taken to represent the actual one Filled and open symbols denote values obtained in hypo- and hyperosmotic media respectively

a simple exponential with a mean rate constant of approximately *0.034 min⁻¹* a figure that has been taken to represent the rate of transmembrane efflux of cellular ionized sodium The extrapolated intercept with the ordinate of this exponential gave the same value for $[Na^+]_i$ as that calculated above 15.7 meq/l cell water However, this figure must be corrected according to the formula deduced by Huxley (see Jonsson 1971) due to interaction between the exponentials representing decrease in extracellular and cellular radioactivity The values finally obtained for the $[Na^+]_i$ concentration and its efflux rate constant in normal Krebs solution at 15 C is 13.1 ± 0.9 (n = 8) meq/l cell water and 0.0339 ± 0.0009 min^{-1}, respectively

The triangles and squares of Fig 6 represent similarly treated Na washout curves obtained in Krebs minus 30 mmoles NaCl/l 230 mOsm/kg and in Krebs plus 150 mmoles sucrose/l (444 mOsm/kg, respectively The values for $[Na^+]_i$ and its efflux rate constant were for the hypotonic medium 17.2 ± 1.2 meq/l, 0.0656 ± 0.0019 min^{-1} and for the hypertonic 16.1 ± 0.7 meq/l, 0.0589 ± 0.0018 min^{-1}, respectively

TABLE I

Incubation medium	Time in minutes of exposure to ^{24}Na	^{24}Na space ml/100 g wet weight Mean ± SE	n	P_{max}	Corresponding ^{14}C sucrose space ml/100 g wet weight Mean ± SE	n	P_{max}
Normal Krebs	10	60 7 ± 0 9	11	0 9	52 8 ± 0 9	13	0 5
	30	60 5 ± 0 5	10		53 9 ± 0 6	12	
Krebs — 30 mmoles NaCl/l	10	58 3 ± 0 7	9	0 5	50 5 ± 0 8	8	0 7
	30	59 6 ± 1 2	5		51 3 ± 1 2	12	
Krebs + 150 mmoles sucrose l	10	69 7 ± 0 2	4	0 3	59 8 ± 1 2	6	0 8
	30	70 6 ± 0 6	4		59 5 ± 0 4	6	

3 *Uptake of ^{24}Na* Due to the action of the postulated sodium-potassium pump, information concerning the passive permeability characteristics of cell membranes is most reliably obtained from washout experiments with respect to potassium but from uptake experiments for sodium For this reason the sodium washout experiments were completed with studies on the uptake kinetics of ^{24}Na at 15° C and at different osmolalities The periods of exposure to ^{24}Na were 10 and 30 min To obtain conditions as comparable as possible with those of the washout experiments, the temperature was lowered to 15° C and the osmolality was changed 30 and 15 min, respectively, prior to the exposure to active solution Since, however, the greater part of the sodium of the muscle is contained in the extracellular space, it was considered necessary to determine the actual ^{14}C sucrose space as well (see above) These determinations were performed on veins that had been exposed to the low temperature and to the anisosmotic solution for periods of time comparable to those applied in the ^{24}Na uptake experiments The results are summarized in Table I

After the initial 10 min, the cellular uptake of ^{24}Na was insignificant for the subsequent 20 min period Values for the rate and magnitude of the influx could therefore not be calculated However, since there was no statistically significant change in the ^{24}Na space of the tissue during this period it may be concluded that $[Na^+]_i$ was then largely constant implying that inward and outward Na fluxes were of equal magnitude If so, it would be possible to use data from the Na washout studies to calculate the passive permeability coefficients

Discussion

The influence of altered extracellular osmolality on the spontaneous electrical and mechanical activity of the rat portal vein, as well as on its cell volume and ionic composition, has been investigated in earlier studies (Mellander *et al* 1967, Joha son and Jonsson 1968 [illegible]

correlation between cell volume and degree of spontaneous activity of the vascular smooth muscles was demonstrated—cell shrinkage being associated with inhibition of spontaneous activity and *vice versa*. These osmolality effects on muscle activity, particularly those of hypo osmolality, could not be accounted for only by the concomitant changes in the transmembrane ionic gradients (Mellander *et al.* 1967, Johansson and Jonsson 1968, Jonsson 1969 a).

For such reasons it was suggested that also the *permeability characteristics of the* cell membranes may be altered by osmotically induced swelling or shrinkage of the smooth muscle cells. For example, an increase (decrease) in the P_{Na}/P_K ratio on exposure to hypo osmotic (hyperosmotic) solutions would potentiate the depolarizing (hyperpolarizing) effect of the concomitant alterations in the transmembrane ionic gradients (Mellander *et al.* 1967, Johansson and Jonsson 1968, Jonsson 1969 a). This hypothesis was indirectly supported by the finding that the excitation caused by hypotonic media could be almost abolished if $[Na^+]_o$ was reduced from 137 to 32 meq/l (Jonsson 1969 a). Consequently it seemed to be of interest to investigate more directly whether alterations in cell volume induce changes in the P_{Na}/P_K ratio, which was the aim of the present study.

The rate constants for the cellular efflux of ^{42}K and ^{24}Na at different extracellular osmolalities, reported in the result section above, do not *per se* indicate any conspicuous change in membrane permeability. It is however still possible that P_K and P_{Na} were to some extent affected by the osmolality shifts because alterations in other factors, such as cell volume and membrane potential, might have cancelled out their influence on the rate constants. Therefore, a more detailed analysis of the results involving calculations of the permeability coefficients for sodium and potassium is required.

Calculations of the permeability coefficients

The formula presented in the concomitant study (Jonsson 1971) for calculation of permeability coefficients (P) for uncharged particles gives

$$P = \frac{V}{A} \cdot \lambda \tag{1}$$

where V/A stands for the volume to surface ratio of the cells and λ for the rate constant of the transmembrane efflux. However, this formula cannot be directly applied to ions since the electrical field across the cell membrane opposes or assists their transmembrane passage. A correcting factor for this influence must therefore be included in the formula. If one applies the simplifying constant field assumptions used by Goldman (1943) this factor is for univalent ions

$$\frac{EF/RT}{1-\exp(-EF/RT)}$$

E stands for the potential difference across the membrane. The sign for E is taken as positive when the ionic movement is assisted and negative when it is opposed by the

electrical field. F denotes Faraday's constant, R the gas constant and T the absolute temperature. The formula for calculations of P values for univalent ions should then be written

$$P = \frac{V\lambda}{A \dfrac{EF/RT}{1-\exp(-EF/RT)}} \tag{2}$$

The membrane potential of the portal vein smooth muscles has not been measured in the present study but has been reported by other authors to average 50 mV in normal Krebs solution (Nakajima and Horn 1967). With this value for E, the correction factor for outward movements of univalent cations amounts to 0.32 at 37° C. For taenia coli the membrane polarization decreases with temperature with a Q_{10} of 1.3 in the interval 32—22° C (Bülbring and Kuriyama 1963). If this relation holds true also for the portal vein, where direct measurements are lacking, the membrane potential would here be about 30 mV at 15° C. However, while the spontaneous discharge of taenia coli ceases at 20° C, the portal vein still displays rhythmic activity at 15° C. The vascular smooth muscle cells may therefore be expected to be less depolarized on temperature reductions and a figure of 40 mV seems fairly reasonable for the membrane potential of the portal vein in normal Krebs solution at 15° C. If so, the correction factor for outward movements of univalent cations would be 0.398 and for inward movements 2.02. The utilized V/A ratios at the different osmolalities are taken from the concomitant article (Jonsson 1971).

Since the efflux of an ion (Mo) according to Keynes and Lewis (1951) is given by the equation

$$Mo = \lambda \cdot C_i \cdot V/A \tag{3}$$

where C_i stands for the intracellular ionic concentration and λ for the rate constant of the efflux, equation 2 can be rewritten as

$$P = \frac{\text{Efflux}}{C_i \cdot 0.398} \tag{4}$$

When permeability coefficients (P) for monovalent cations are calculated from *influx* experiments at 15° C the equation will be

$$P = \frac{\text{Influx}}{C_o \cdot 2.02} \tag{5}$$

where C_o stands for the extracellular concentration of the actual ion.

When these formulas are applied to the potassium washout in normal Krebs, the efflux and P values amount to 4.1 · 10^{-12} M/cm² sec and 9.4 · 10^{-8} cm/sec, respectively, at 37° C. At 15° C the corresponding figures are 2.0 · 10^{-12} M/cm² sec and 5.3 · 10^{-8} cm/sec.

As mentioned above, the permeability coefficients for sodium should be calc[ulated] from the Na^+ *influx*. The Na^+ efflux in normal Krebs at 15° C amounts to

M/cm² sec and, since the uptake experiments presented in section 3 indicated steady state regarding $[Na^+]_i$ during the corresponding washout period, it might be justified to assume that the Na^+ influx is about the same With these assumptions a P_{Na} value of 4 3 10^{-8} cm/sec is obtained, giving a P_{Na}/P_K ratio of 0 081 at 15° C

The effluxes of potassium and sodium in M/cm² sec in Krebs plus 150 mmoles sucrose/l amount to 2 5 10^{-12} and 1 3 10^{-12}, respectively, and in Krebs minus 30 mmoles NaCl l to 2 5 10^{-12} and 2 0 10^{-12}, respectively Again the calculations of the corresponding P values are uncertain because the actual membrane potential and hence the correction factor is unknown For this reason, P values have been computed for three different membrane potentials (40, 50 and 60 mV in the hypertonic and 40, 30 and 20 mV in the hypotonic solution) and expressed in per cent of P_K and P_{Na} in normal Krebs

The values are summarized in Fig 7 P values for Na are represented by squares, for K by points Filled and open symbols denote values obtained in hypo and hyper osmotic media, respectively A comparison of these different P values suggests that P_{Na} increases more than P_K on reductions in the extracellular osmolality, giving an increased P_{Na}/P_K ratio This increase may be exaggerated by the surprisingly high value for $[Na^+]_i$ obtained in the hypo-osmotic solution However, P_{Na} values were also computed on the assumption that the total amount of cellular sodium remained constant during osmotic swelling and that therefore $[Na^+]_i$ decreased somewhat due to the slight swelling but even then P_{Na} (filled triangles) increases more than P_K

The interpretation of the effects of hyperosmolality on P_K and P_{Na} is more intriguing since the membrane potential is then of critical importance A rough estimate, based on the "constant field equation" concerning the hyperpolarization that would be caused by the addition of 150 mmoles sucrose/l yields a value around 10 mV In the taenia coli Tomita (1966) found an increase in the membrane potential of 10—15 mV on exposure to twice tonicity Krebs solution A hyperpolarization of this order of magnitude will according to the data summarized in Fig 7, not markedly influence either P_K or P_{Na}

The calculations of the percentual changes in P_{Na} and P_K obtained on exposure to anisosmotic media in the present study, were based on the assumption that the membrane potential averaged 40 mV in normal Krebs solution at 15° C It can, however, easily be demonstrated that approximately the same relative effects are obtained irrespective of whether the membrane potential in isotonic solution is taken as 30, 40 or 50 mV

For comparison calculations of P_K values were performed with the assumption that the part of the ^{42}K washout curve in the interval 60—120 min represented the transmembrane exchange of potassium at 15° C (see Fig 1) This analysing method gave lower estimates for P_K and therefore a higher P_{Na}/P_K ratio (0 23) The estimated percentual alterations in these P_K values on exposure to lowered osmolality were almost identical with those given in Fig 7 deduced from the earlier part (7—30 min) of the K-efflux curve However, the similarly calculated P_K values for the hyperosmotic situation were, in contrary to those given above, considerably higher than

the corresponding control value, an observation that no doubt could explain the hyperpolarization obtained in these media Yet, it would be difficult to understand the reason for such a pronounced raise in P_K when the cells are shrunken In the following discussion, only the interpretation of the potassium exchange presented in the preceding sections will be regarded

After these calculations the present results concerning distribution and exchange of Na and K will be considered in the light of previous studies of similar problems in smooth muscle

A survey of the literature concerning the ionic composition of smooth muscle reveals considerable uncertainty concerning the $[Na^+]_i$ values The pronounced scattering in the values reported seems to depend on dissimilarities in the methodological approach, on differences in the estimate of the magnitude of the bound fractions and, when $[Na^+]_i$ has been deduced from washout experiments, on the difficulty to ascertain what part of the curve that represents exchange of intracellular sodium Thus, for guinea pig taenia coli estimated values for $[Na^+]_i$ range from 13 mmoles/l cell water (Casteels 1969) to 56—85 mmoles/l (Goodford and Hermansen 1961) For arterial smooth muscle estimated $[Na^+]_i$ values range from 7 mmoles/l (Keatinge 1968) to 50 (Garrahan, Villamil and Zadunaisky 1965) and 84 mmoles/l (Hagemeijer, Rorive and Schoffeniels 1965 a)

In an earlier study (Haljamae *et al* 1970) the ionic composition of intracellular fluid in the portal vein was estimated by subtracting the extracellular and bound amounts from the total ionic content determined by flame photometry The $[Na^+]_i$ values thus obtained were, however, considerably higher than those extrapolated from the washout curves in the present study (34—45 compared to 13 mmoles Na^+/l) but this is a common finding also in other kinds of smooth muscle when the results with the two methods are compared For example the higher values in the papers quoted above were obtained with flame photometry

Most authors seem to agree that a significant sodium fraction prevails in a "bound", osmotically inactive form, since $[Na^+]_i$ values computed from a simple two compartment model may even exceed $[Na^+]_o$ (Headings Rondell and Bohr 1960, Friedman and Sreter 1963) As to the rat aorta Rorive (1969) found evidence of five different sodium fractions of which at least two would represent bound sodium The present experiments revealed at least three components in the ^{24}Na washout curve of which the one with the lowest rate constant was taken to represent intracellular, "bound" sodium, deduced to make out 0 3 meq/kg wet weight However, since the total amount of bound sodium in the portal vein was calculated to exceed 10 meq/kg wet weight in the earlier study (Haljamae *et al* 1970), a far larger "bound" fraction, washed out together with $[Na^+]_o$ may exist In fact, such a rapidly exchanging bound Na fraction has been demonstrated in dog carotid arteries (Garrahan, Villamil and Zadunaisky 1965) and in rat aorta (Schoffeniels 1969) A small slowly exchanging Na fraction, such as that found for the rat portal vein in the present study, has been reported for the guinea pig taenia coli (Durbin and Jenkinson 1961, Goodford 1962, Buck and Goodford 1966 Bulbring Goodford and Setekleiv 1966, Brading and Jones 19

as well as for different kinds of arteries (Garrahan, Villamil and Zadunaisky 1965, Hagemeijer, Rorive and Schoffeniels 1965 b) Different interpretations of this slowest component have been proposed

In this context it should be mentioned that it is not generally agreed that a distinct component of the sodium washout curve can be taken to represent exchange of intracellular ionized Na Brading and Jones (1969) demonstrated for taenia coli that 98—99 per cent of the ^{24}Na exchange could be described by bulk diffusion kinetics thus suggesting that the cell membranes may not act as rate limiting barriers However, the fact that changes in temperature or exposure to ouabain markedly influenced the component of the washout curve, considered to represent exchange of cellular sodium in the taenia coli by Buck and Goodford (1966) and in the arterial smooth muscle by Garrahan, Villamil and Zadunaisky (1965) and Hagemeijer, Rorive and Schoffeniels (1965 b) indicates the existence of a membrane function It might therefore be justified to assume that, at least in some situations, the $[Na^+]_i$ exchange should appear as a clearcut single exponential

The present value for Na efflux in the portal vein ($1.2 \cdot 10^{-12}$ M/cm^2 sec) is somewhat lower than those reported for the taenia coli ($3.0 \cdot 10^{-12}$ M/cm^2 sec, Buck and Goodford 1966, $7.2 \cdot 10^{-12}$ M/cm^2 sec, Casteels 1969) However, the difference may at least in part, be ascribed to the fact that the latter values were obtained at 35° C while that for the portal vein was acquired at 15° C Keatinge (1968) gave a still lower value ($0.18 \cdot 10^{-12}$ M/cm^2 sec) for sheep carotid arteries and attributed the discrepancy to the fact that the carotid artery in contrast to the taenia coli (and portal vein) is not spontaneously active

The present calculation of the Na permeability constant for the portal vein was based on the assumption that the Na influx largely equalled the efflux, *i e* that $[Na^+]_i$ remained constant during the washout of ^{24}Na Though taenia coli smooth muscle gains sodium and loses potassium when temperature is lowered (Freeman—Narrod and Goodford 1962, Buck and Goodford 1966), the cells are, even at 20° C able to extrude excess sodium accumulated during the preparation and thus reach a new level of equilibrium concerning *e g* $[Na^+]_i$ (Freeman—Narrod and Goodford 1962) The portal vein cells may therefore well be capable of maintaining $[Na^+]_i$ at a fairly constant level for a limited period of time even at 15° C, a view supported also by the ^{24}Na uptake experiments summarized in Table I

The ^{42}K washout at 37° C formed after some minutes a simple exponential whose extrapolated intercept with the ordinate corresponded to a $[K^+]_i$ of 137 meq/l in agreement with the results of flame photometry (Haljamae *et al* 1970) A similar agreement in $[K^+]_i$ values has been reported for rat aorta (Hagemeijer, Rorive and Schoffeniels 1965 a, b) Though the existence of bound fractions in the smooth muscle has been reported also for potassium (Buck and Goodford 1966, Goodford 1966, Haljamae *et al* 1970) the relative magnitude of bound K, compared to free cellular K^+, seems to be smaller than in the case of sodium This might be an important reason for the better reproducibility in the $[K^+]_i$ values Another factor of sig-

nificance in this context is that potassium, in contrast to sodium, is mainly located intracellularly and therefore less influenced by differences in the blotting procedure

When the ^{42}K washout was performed at 15° C the curve was not strictly monoexponential until after one hour The reason for this might be that the passive permeability of the cells decreases slowly on longer exposures to 15° C However, in most cases the washout during the interval 7—30 min had a nearly monoexponential shape, indicating fairly stable permeability conditions also during this earlier period

The K efflux value at 37° C, calculated for the portal vein (4 1 10^{-12} M/cm^2 sec), conforms closely with those given for the taenia coli (3—5 10 ^{12} M/cm^2 sec, Freeman Narrod and Goodford 1962, 2—4 10^{-12} M/cm^2 sec, Goodford and Hermansen 1961, 4 1 10^{-12} M/cm^2 sec Casteels 1969) As to the effect of temperature on the taenia coli, Setekleiv (1967) reported a Q_{10} of 1 94 in the interval 10—40° C for the increase in the rate of K efflux The present values of reduced K efflux upon lowering the temperature from 37° C to 15° C was somewhat less pronounced for the portal vein than for the taenia coli

The "passive permeability constants, P_K and P_{Na}, have been calculated on the assumption that the Na influx and K efflux are *per se* independent and subjected only to electrical and concentration gradients The P_{Na}/P_K ratio of 0 081 calculated above, as well as the value of 0 12 estimated in the earlier study (Haljamae *et al* 1970), fall within the range reported by Buck and Goodford (1966) and Casteels (1969) for the taenia coli (0 04—0 07 and 0 16, respectively)

It was recently shown that exposure of the taenia coli to hyperosmolality increased the rate constants for $[K^+]_i$ and, probably, for $[Na^+]_i$ in the washout curves in contrast to that of chloride (Brading 1970) It was proposed that this could be due to the concomitant increases in $[Na^+]_i$ and $[K^+]_i$ and that P_{Na} or P_K did not undergo any more pronounced change This is in general agreement with the present results on hyperosmolality effects on the portal vein, as summarized in Fig 7, while hypo-osmolality seems to cause a clearcut increase of the P_{Na}/P_K ratio Thus, these latter findings support the hypothesis that hypo osmolality excites vascular smooth muscle both by increasing the membrane permeability to the sodium ion and by affecting the transmembrane ionic concentration gradients

It should be stressed, however, that these findings may be only indicative since the assumption that the influx and efflux of sodium in normal as well as in anisosmotic Krebs solutions are equal in magnitude during the washout period may not be fully justified Furthermore the calculations of the efflux values are subjected to the uncertainties associated with the interpretation of the washout curves It might also be objected that the increased rate of sodium exchange observed in the hypotonic media may not be the cause of the excitation but merely a consequence of it, due to the increased spike activity, provided that the inward current during the action potentials is carried by the Na ion However there is experimental evidence that the action potential in smooth muscle may be mediated by calcium rather than by sodium (Nonomura Hotta and Ohashi 1966 Brading and Tomita 1968, Brading and Tomita 1969) If this is also so for the portal vein, the increased N

hypo-osmotic media would not be a secondary phenomenon to the excited electrical activity

In summary, the present results support the idea that the excitation caused by hypo-osmolality is partly mediated *via* an increased P_{Na}/P_K ratio, whereas no such mechanism could be demonstrated for the responses to hyperosmolality, but could neither be excluded However, since the results of previous studies clearly indicate that the effects of hyperosmolality cannot be fully explained by the altered ionic gradients, it is possible that some other type of mechanism is involved The observation made by Sperelakis and Schneider (1968) that the chloride conductance/potassium conductance ratio of the frog sartorius muscle decreases on increases in tonicity is of interest in this connection, because the Cl^- equilibrium potential is considerably less negative than the membrane potential of the portal vein (Haljamae *et al* 1970) A decreased Cl^- influence on the membrane potential during exposure to hyperosmotic solutions might then contribute to the hyperpolarization of the smooth muscles, and *vice versa* during hypo osmolality Furthermore, the possibility that the sodium pump in smooth muscle is electrogenic in nature (Taylor, Paton and Daniel 1969) implies that the membrane potential might be influenced by an osmolality effect on this pump Such effects of osmolality on the active sodium transport in frog epithelium have been reported (Ussing 1965)

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In summary, the present results support the idea that the excitation caused by hypo-osmolality is partly mediated *via* an increased P_{Na}/P_K ratio, whereas no such mechanism could be demonstrated for the responses to hyperosmolality, but could neither be excluded However, since the results of previous studies clearly indicate that the effects of hyperosmolality cannot be fully explained by the altered ionic gradients, it is possible that some other type of mechanism is involved The observation made by Sperelakis and Schneider (1968) that the chloride conductance/potassium conductance ratio of the frog sartorius muscle decreases on increases in tonicity is of interest in this connection, because the Cl^- equilibrium potential is considerably less negative than the membrane potential of the portal vein (Haljamäe *et al* 1970) A decreased Cl^- influence on the membrane potential during exposure to hyperosmotic solutions might then contribute to the hyperpolarization of the smooth muscles and *vice versa* during hypo-osmolality Furthermore, the possibility that the sodium pump in smooth muscle is electrogenic in nature (Taylor, Paton and Daniel 1969) implies that the membrane potential might be influenced by an osmolality effect on this pump Such effects of osmolality on the active sodium transport in frog epithelium have been reported (Ussing 1965)

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From the Department of Physiology, University of Goteborg, Sweden

Motor Unit Activity and Stiffness of the Contracting Muscle Fibres in the Tonic Stretch Reflex

By

S GRILLNER and M UDO*

Granit (1958) pointed out that the length tension curve of the contracting muscle fibres could well contribute to the stiffness of the tonic stretch reflex as does the recruitment of new motor units The present investigation was undertaken to find *the normal rate of motoneuronal firing in the homogenous soleus muscle during the* tonic stretch reflex in the intercollicularly decerebrated cat and to investigate the relation of length and tension in a deafferented contracting muscle with spontaneous α-motoneurone discharge at frequencies comparable to those found in the decerebrate preparation

The soleus muscle was freed 1
d cutting the tendon of the
was measured at minimal and
with its bony insertion from th
soleus muscle was dissected fre
bony insertion to a strain gauge (compliance 150 μ/kg) The distal and proximal parts of the tibia were rigidly fixed The muscle was extended (and released) at a low constant velocity (0 8 mm/sec) to the maximal muscle length in situ The activity of single motor units was sampled by recording on the muscle surface with small spring mounted glass microelectrodes (3M NaCl resistance 0 5—2 MΩ) or tungsten electrodes (resistance 100—200 KΩ The motor unit activity was directly recorded on film and the discharge pattern was analyzed by hand This method has the advantage of sampling the activity of units without interfering with the reflex control of the individual motor units

The instantaneous frequency of one motor unit during slow stretch at constant velocity (0 8 mm/sec) up to maximal physiological extension *i e* corresponding to a joint angle of 30° in the ankle is shown in Fig 1 A The unit was recruited 6 mm before maximal extension and the first few spikes occurred at a low frequency but then the frequency was maintained at a remarkably constant rate (9 1 imp/sec throughout the slow extension As soon as the muscle was held at the maximal length the frequency dropped to 8 2 imp/sec which was again maintained at a constant level The drop in frequency after the termination of the ramp stretch was always found but rarely exceeded 2 imp/sec The frequency of discharge during dynamic extension was always maintained at a nearly constant rate except for minute in creases (<0 4 imp/sec) *which were sometimes encountered* The impulse frequency could then also be increased by other excitatory inputs such as repetitive nerve stimu

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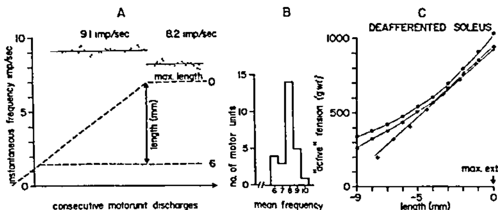


Fig 1 *A* is a graph of the instantaneuos frequency (1/interspike interval) of successive action potentials in one soleus motor unit during a continuous slow stretch of the soleus muscle followed by maintained extension The motor unit was active for the last 6 mm of the extension *B* shows a histogram of the mean frequency for 26 soleus motor units during maintained extension at the maximal physiological extension. *C* is a graph of the "active" tension developed by the deafferented (dorsal roots cut) soleus muscle of two different preparations (dots and crosses respectively) versus the muscle length during slow extension (0.8 mm/sec) up to maximal physiological extension The passive tension, *i.e.* the tension developed by the muscle without any contraction was subtracted from the total tension in order to obtain the "active"

lation (med. gastrocn. at gr. I strength or contralateral nerves) Hence it appears as if during the dynamic extension at constant velocity (0.8 mm/sec) not enough net excitation is added with increasing length to cause a continuously increasing impulse frequency This discharge pattern would be expected if the Ia afferents were the main excitatory input from the extended muscle

In the histogram of Fig 1 B is shown the discharge frequency of 26 motor units during maintained extension at maximal physiological length of the soleus muscle The average frequency is 7.8 ± 1.0 (S.D.) hence the soleus motor units appear to fire in a rather narrow band

In order to study the stiffness (tension versus length) of the contracting muscle fibres preparations with a resting discharge in the *α* motoneurones were needed for the analysis (*cf. legend*) The graph of Fig 1 C shows the "active" tension (*cf.* legend) developed by the contracting muscle fibres in the deafferented soleus muscle during slow extension of the muscle in two different preparations The EMG activity as judged by the integrated EMG was at a constant level throughout the different extensions and sampled motor units fired between 7.5—9.5 imp/sec *i.e.* in the same range as in the tonic stretch reflex The tension (Fig 1 C) increases monotonically up to maximal physiological length with a steep slope of between 80—90 g wt/mm for the last seven mm It follows that the contracting muscle fibres would act to m-

crease the stiffness of the tonic stretch reflex for increasing muscle lengths over the entire physiological range in which the tonic stretch reflex can be studied in the decerebrate cat

In view of this steep and rather linear relation between length and "active" tension of the muscle fibres and the wellknown linear stiffness in the tonic stretch reflex (Granit 1958), it would have been surprising if there was a linear recruitment in the stretch reflex *i e* that a fixed number of motor units would be recruited for each mm as discussed by Granit (1958) Instead Grillner and Udo (unpubl) have found that the recruitment is in fact highly non-linear with a marked recruitment initially at low tensions and a less pronounced recruitment at higher levels

The recent postulate by Matthews (1969) that the secondary endings contribute to the tension in the tonic stretch reflex rests on the assumption that the length tension curve of the contracting muscle fibres is flat for the last 7 mm (*i e* gain 0 g wt /mm) (*cf* Grillner 1970 Matthews 1970) The present results (Fig 1 C) show on the contrary, that the slope is steep [illegible] at the discrepancy that Matthews (1969) [illegible] the extension (*cf* Fig 1 A) also strongly supports [illegible] main excitatory input in the tonic stretch reflex of the intercollicularly decerebrate cat

The present results thus show that the inherent properties of the contracting muscle fibres in the soleus muscle contribute quantitatively to a large extent in the increase in stiffness of the tonic stretch reflex with increasing muscle length in the range up to maximal physiological extension and further suggest that the Ia afferents are the main excitatory input in the stretch reflex which can recruit the α motoneurones provided that the background excitation is high enough and that the transmission in homonymous inhibitory reflex arcs is depressed (*cf* Grillner and Udo 1970)

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Effect of Bleeding on Plasma Sulfation Activity in Rats

By

HARRY BOSTROM, LJUBOMIR BOŽOVIĆ and MILICA BOŽOVIC

Plasma of normal rats stimulates uptake of sulfate in cartilage of hypophysectomized rats and its incorporation into chondroitin sulphate Addition of growth hormone *in vitro* to cartilage from hypophysectomized rats has no effect on the sulfate uptake Injection of growth hormone into hypophysectomized rats restores the decreased sulfation activity of their plasma From these data it was concluded that the sulfation activity of plasma is growth hormone dependent (Salmon and Daughaday 1957) Homogenates of different tissue such as liver, brain, muscle, heart lung exhibit sulfation activity (Bostrom and Månsson 1953) After chromatography on Dowex 50 of water extracts of skeletal muscle followed by gel filtration on Sephadex G 25 sulfation activity appeared in fractions of low molecular weight (Božovic *et al* 1970) Practically nothing is known about the physiological variations of plasma sulfation activity in normal animals, about the origin of the plasma sulfation activity or the mechanism controlling release of the sulfation factor from tissue into plasma However, many conditions especially stress cause a rapid and marked increase of growth hormone concentration in the plasma of men and animals (Knobil and Meyer 1968) In the present investigation the sulfation activity of plasma was measured under stress The stress chosen was standardized bleeding in anesthetized rats

The experiments were performed on 15 albino rats of both sexes 200—300 g b w The animals were anesthetized by an i p injection of pentothalsodium (30 mg/kg b w) One carotid artery was prepared and a polyethene catheter filled with heparin was placed in the artery The animals were bled via catheter at intervals of 15 min The volume of blood taken in each bleeding as 1 4 ml per 100 g b w Blood was allowed to run freely into a calibrated tube immediately centrifuged and plasma stored until used From each animal three samples of plasma were obtained

Sulfation activity of plasma was tested on the embryonic chick pelvis (Hall and Božović
... °C in 2 ml
... led After
... the subse-
... n activity
of one plasma sample were made the activity of each sample being expressed as the mean of
... washed with saturated nonlabelled
... with water with absolute ethanol
... vacuum dessicator, the dry pelvis
... Permablend was added (Packard
Instrument Co The radioactivity was measured in a fluid scintillation counter

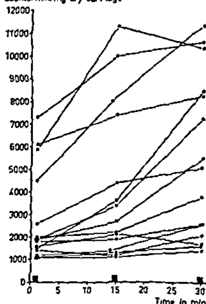


Fig 1 Plasma sulfation activity in rats subjected to bleeding (1 4 ml blood was taken per 100 g b w) as indicated by the squares on the base line

The results obtained in the experiment in which 15 rats were subjected to bleeding are shown in Fig 1 As seen in the figure sulfation activity increased in 12 out of the 15 animals There are considerable variations between animals before bleeding but the general trend of increasing sulfation activity with successive bleedings is quite evident

The statistical analysis of the results is shown in Table I

The present results indicate that bleeding increases the plasma sulfation activity The mechanism of this phenomenon cannot yet be explained Possibly growth hormone released by bleeding causes the increase of plasma sulfation activity This is supportd by a comparison of our results with those obtained by Knobil and Meyer who demonstrated an increase of growth hormone concentration in monkey blood after bleeding On the other hand work of Salmon and Daughaday (1957) indicated

TABLE I Effect of bleeding on plasma sulfation activity in rats as measured by the incorporation of ^{35}S labelled sulfate in embryonic chick cartilage

Number of rats	Counts/min/mg dry cartilage		
	before bleeding	after the 1st bleeding	after the 2nd bleeding
15	2806 (100 %)	4141 (144 %)	5528 (197 %)
MD ± SE	1336 ± 378		1387 ± 422
p	<0 01		<0 01

that sulfation activity appears in plasma as late as ten hours after injection of growth hormone in hypophysectomized rats, and this seems to contradict any hypothesis that growth hormone itself releases the sulfation factor from tissues Other non specific mechanism of release, as for instance hypoxia in bleeding cannot therefore be excluded Whatever the ultimate explanation of our results is they are of immediate practical significance in studies of sulfation activity during which repeated blood samples are drawn from small animals because the bleeding itself might influence plasma sulfation activity

In summary, the results of the present study strongly suggest that the plasma sulfation activity increases with successive bleeding

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Acta physiol. scand. 1971. 81. 428–430
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Morphological Identification of Renshaw Cells

By

E JANKOWSKA and S LINDSTROM

Renshaw cells have never been unequivocally identified although their location has been established in physiological studies (Renshaw 1946, Eccles, Fatt and Koketsu 1954, Thomas and Wilson 1965) to be at the medio-ventral border of the motor nuclei where Szentagothai found terminal branching of motor axon collaterals (1967) as well as cells which might be Renshaw cells (1958) An attempt to stain these cells by intracellular iontophoretic injection of methyl blue or fast green (Erulkar *et al* 1968) was not successful and the elements stained could not be readily identified as cell bodies

Using Procion Yellow ICI (Stretton and Kravitz 1968) for intracellular injection we have now been able to stain 8 Renshaw cells The electrodes used had tips broken to 1 5–2 0 μ and were filled with a 6 % solution of Procion Yellow The dye was ejected by a 5–10 nA constant hyperpolarizing current during 3–15 min Otherwise the procedure was as described previously (Jankowska and Lindstrom 1970) The Renshaw cells were indentified by their typical high frequency discharge to stimulation of the ventral roots (Renshaw 1946) as shown in Fig 1



Fig 1 Photomicrograph of a Renshaw cell lightly stained with Procion Yellow The bar corresponds to 10 μ The records to the right (upper traces intracellular potentials lower traces cord surface potentials were taken just before the intracellular injection of the dye The time calibration is 1+5 msec for the upper records and 10+50 msec for the lower ones the voltage calibration is 10 mV and refers to intracellular potentials The location of the cell is indicated by a circle in the diagram to the left A triangle and points show location of the Renshaw cell in Fig 2 and of 4 other Renshaw cells in L7 Shaded extent of motor nuclei



Fig 2 A montage of photomicrographs from 3 successive slides, 15 μ thick showing soma dendrites and axon (arrows) of another Renshaw cell The bar in the right corner corresponds to 20 μ The picture is oriented so that the right side is medial and parallel with the midline

Fig 1 and 2 show photomicrographs of two Renshaw cells That in Fig 1 was stained lightly, showing the nucleus and contours of the soma and proximal dendrites The cell in Fig 2 was stained more heavily, revealing the dendritic tree and the proximal part of the axon (arrows) The latter was identified by its uniform diameter (after the very thin initial part) and by a gradual weakening of the intensity of the fluorescence typical for axons (unpublished observations *cf* Fig 1 in Jankowska and Lindstrom 1970) The Renshaw cells seem to have a diameter of 10—15 μ and closely resemble the cell illustrated by Szentágothai (1958 Fig 5) The dendrites reconstructed in 2 cells, extended to a radius of about 100—150 μ some going out in the white matter All stained cells were located at the medio-ventral border of the motor nuclei (Fig 1), though none of them were in close proximity to motoneurone somas (*cf* Erulkar *et al* 1968)

It is of particular interest that in 2 Renshaw cells whose axons were traced for about 200 and 400 μ respectively one of the axonal branches could be followed to the border of the ventro medial funiculus This indicates that the Renshaw cells may be funicular neurones as suggested by Scheibel and Scheibel (1966) and Szentagothai (1967) It would also agree with the postulated intersegmental inhibitory actions of the Renshaw cells on other Renshaw cells in the neighbouring segments (Ryall 1970), on Ia interneurones (Hultborn *et al* 1971) and on motoneurones (Hultborn, Jankowska and Lindstrom to be published) located one or two segments away from the location of the Renshaw cells

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Acta physiol scand 1971. 81. 431—432
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On the Nature of the Synthesis of Adenosine Triphosphate at the Surface of Intact Tumour Cells

By

G Ågren and G Ronquist

Tumour cells are known to have an increase of anaerobic glycolysis (Warburg 1956) In a series of papers the ability of the plasma membrane from human erythrocytes and tumour cells to synthesize ATP has been studied (Ronquist and Ågren 1966 Ronquist 1969 Ågren and Ronquist 1969 Ågren *et al* 1971) This ATP is formed in the first energy yielding step of glycolysis involving the glyceraldehyde 3 phosphate dehydrogenase (E C 1 2 1 12) and phosphoglycerate kinase (E C 2 7 2 3)

It has previously been shown that neither ADP, NAD nor 3 GAP inside the cell can be used outside in reactions on the surface of the red cell membrane (Ronquist 1969) Neither could the ATP synthesis be explained by an exchange reaction between (^{32}P) orthophosphate and the ATP formed in the adenylate kinase reaction (Ronquist 1969)

In the present paper it has been demonstrated that extracellular labelled ATP could be isolated after incubating tumour cells under the same conditions as used in the erythrocyte experiment Calculated per dry weight of cells the tumour cells were able to form higher amounts of labelled ATP than the erythrocytes (Ågren and Ronquist 1969) There was no reason to believe that the reaction of the tumour cells should be of the exchange type but necessary experiments were not carried out until now The results given in the present paper show that such a reaction could be excluded in the experiments with tumour cells

Ehrlich tumour cells were used Experimental conditions are given in details in a previous paper (Ågren and Ronquist 1969) The results are given in Table I It is evident from the data that all substrates and cofactors of the glyceraldehyde 3 phosphate dehydrogenase and phosphoglycerate kinase are necessary for synthesis of labelled ATP Tumour cells incubated in the incomplete medium formed only negligible amounts of labelled ATP Similar small amounts were found when the tumour cells were incubated with the complete medium lacking one of the cofactors (ADP NAD or 3 GAP)

Abbreviations ATP adenosine triphosphate ADP = adenosine d phosphate NAD = oxid zed nicotinam de d nucleotide 3 GAP = glyceraldehyde 3 phosphoric acid

TABLE I Total (^{32}P)ADP and (^{32}P)ATP formation by Ehrlich ascites tumour cells on incubation with the complete and incomplete systems, as well as with the complete system lacking only one cofactor

	(^{32}P)ADP	(^{32}P)ATP
Complete	0 029 (1 81)	0 127 (0 86)
Incomplete	0 001 (0 13)	0 009 (0 54)
Complete minus ADP	0 001 (0 14)	0 008 (0 52)
Complete minus NAD⁺	0 001 (1 83)	0 009 (0 89)
Complete minus 3 GAD	0 001 (1 89)	0 006 (0 81)
Complete minus $MgCl_2$	0 018 (1 80)	0 069 (0 81)

The results given are the incorporation of (^{32}P)orthophosphate (in μmoles) into ADP and ATP per 100 mg dry weight of Ehrlich ascites tumour cells Figures in brackets denote the total amount of ADP and ATP recovered after the incubation period (1 min) expressed as μmoles per 100 mg dry weight of tumour cells The experimental conditions have been given previously (Ågren and Ronquist 1969)

A comparison of the total amounts of ATP formed by tumour cells incubated either in the complete or in the incomplete medium gives a difference of 0 32 μmoles Of this amount 0 13 μmoles represent the *de novo* synthesis of ATP while the remaining part, 0 19 μmoles are formed by adenylatekinase present at the cell surface (*cf* Ågren and Ronquist 1969) Since this enzyme catalyses a dismutation reaction between 2 moles of ADP giving 1 mole AMP and 1 mole ATP it is natural that in all media where ADP is present, an additional amount of ATP can be formed Therefore tumour cells incubated with the complete medium lacking either NAD⁺ or 3-GAP could form an extra amount of ATP in this reaction.

On the other hand the incorporation of (^{32}P) orthophosphate into ATP was not influenced by the presence of ADP in the incomplete medium indicating the necessity of all cofactors to be present in the ATP synthesis Therefore (^{32}P)orthophosphate in the incomplete medium or a complete medium lacking one of the necessary cofactors could not be exchanged for a terminal phosphoryl group of ATP

While the magnesium ions seem to be completely available in erythrocytes (Ronquist 1969) the conditions seem to be different in tumour cells where less labelled ATP was synthesized when magnesium ions were excluded from the medium

We are indebted to Ing S Eklund for skilful technical assistance This investigation was supported by grants from the Swedish Cancer Society (project No 290 B69 01X) and the Medical Research Council Project No B71 13X 228 07C)

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Histochemical Demonstration of Carbonic Anhydrase Activity in the Rat Kidney

By

Gudmar Lönnerholm

Received 16 September 1970

Abstract

Lönnerholm G *Histochemical demonstration of carbonic anhydrase activity in the rat kidney* Acta physiol scand 1971 *81* 433—439

The carbonic anhydrase activity in the kidney of male Sprague-Dawley rats was studied by a histochemical technique Enzyme activity was found not only in the proximal and distal

vestigators have found an increase in the number of the so called dark cells after administration of sodium or potassium bicarbonate to rats

Carbonic anhydrase (carbonate hydrolase, EC 4 2 1 1) activity has been previously demonstrated by various histochemical techniques in the proximal and distal tubules of the rat and mouse kidney (Hausler 1958 Hansson 1968 Ishizaki 1969) Hausler (1958) and Ishizaki (1969) reported that no activity was found in the other parts of the kidney tubules Hansson (1968) only investigated the outer cortical zone The purpose of the present work was therefore to make a systematic search for carbonic anhydrase activity in all parts of the kidney tubules using the recently introduced sensitive histochemical method of Hansson (1967) The knowledge of the distribution of the enzyme is of obvious importance for an understanding of its role in kidney function and the effects of carbonic anhydrase inhibitors

Methods

Male Sprague Dawley rats weighing 300—375 g were used They were fed standard rat pellets (Anticimex 210 Anticimex Evefund Sollentuna, Sweden) and water *ad libitum* The rats were anesthetized with Nembutal® 50 mg/kg injected intraperitoneally

Preparation of tissues

Fixed and unfixed tissue was used

Unfixed tissue

One of the kidneys was perfused in vivo through a polyethylene tube in the abdominal aorta to avoid contamination with enzyme from the erythrocytes The perfusion solution was 0 2 M sucrose in 0 1 M phosphate buffer at pH 7 4 or simply 0 9 % NaCl The perfusion lasted

2—3 min The pressure was 150 cm H_2O and the renal vein had been opened to secure free flow The kidney was then removed and cut in slices 2—3 mm thick which were immediately frozen in isopentane cooled with liquid nitrogen Sections were cut at −20° C and handled in one of two ways

1) 4 μ sections were thawed on a *TH WP Millipore filter 25 μ thick pore size 0 45 μ* (Millipore Filter Corporation Bedford Mass, USA) and then immediately stained

2) 15 μ sections were freeze dried for 1 hr at 0 03 mm Hg and −50° C and then stained These sections could be stored for some days before staining

Fixed tissue

2 5 % glutaraldehyde in 0 1 M phosphate buffer at pH 7 4 was used as fixative The glutaraldehyde was prepared from a 50 % stock solution by a one stage vacuum distillation (Andersson 1967) The purity of the distillate was checked by ultraviolet spectrophotometry One of the kidneys was perfused in vivo with the fixative for 15—20 min as already described The kidney was then removed and cut in slices 2—3 mm thick which were incubated in the perfusion solution for 16 hrs at 4° C The slices were then washed *in 0 2 M sucrose in* 0 1 M phosphate buffer at pH 7 4 for 4 hrs at 4° C to remove excess fixative The slices were thereafter frozen in isopentane—liquid nitrogen and sectioned at −20° C The sections were handled in one of two ways

1) 4 μ sections were thawed on Millipore filters and stained

2) 8 μ sections were collected in a Petri dish containing a cold sucrose phosphate solution and were then stained within 10 min

Staining procedure

The sections were stained for carbonic anhydrase activity according to the method of Hansson (1967 1968) The volume of the incubation medium was 57 ml and always included 10 ml *1/15 M KH_2PO_4 to ensure maximal sensitivity and short incubation times 1—6 min for free* floating sections and 3—9 min for sections on Millipore filters Acetazolamide 10^{-6} M (Diamox® Sodium American Cyanamid Company Pearl River N Y USA) in the incubation medium completely abolished the visible staining both in the glutaraldehyde fixed and the unfixed sections Acetazolamide 20 mg/kg given intravenously 30 min before removal of the kidney gave a delayed and weakened staining reaction Some sections were counterstained with haematoxylin and eosin In some experiments every other section was stained for carbonic anhydrase activity and every other for mitochondria with aniline—acid fuchsin—methyl green according to Lillie (1954) Pairs of sections 4 μ thick were matched in this way

Microphotography

Microphotographs were taken on Kodak Panatomic X film negative size 24×36 mm using a Zeiss standard microscope *GFL* with a Zeiss photo attachment *The built in tungsten lamp* was used A Zeiss lens Plan Achromat 40/0 65 was used together with a Zeiss grey filter 0 03 or a Zeiss oil immersion lens Plan Achromat 100/1 25 was used without any filter

Nomenclature

The different parts of the kidney tubules were named according to Latta Maunsbach and Osvaldo (1967) the convoluted and straight part of the proximal tubule the thin segment is also known as the ascending thick segment of Henle s loop) the arched collecting tubule and the straight collecting tubule See Fig 1 The rat kidney is conveniently divided into four zones according to McFarlane (1941) the outer and inner zone of the cortex and the outer and inner zone *of the medulla The border between cortex and medulla* lies where the proximal tubules change to the thin segments of Henle s loop The inner medullary zone consists of the thin segments of Henle s loop and the straight collecting tubules while the outer medullary zone also contains the straight part of the distal tubules

Results

The preservation of tissue structure was very satisfactory after gluturaldehyde fixation, and the perfusion of fixative caused the lumen of the tubules to remain open The unfixed freeze dried sections were intensely stained but the staining was not

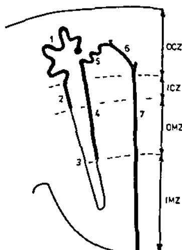


Fig 1 [illegible] -oximal tubule, [illegible] loop, 4) dist[illegible] convo-luted p[illegible] [illegible]t col-lecting [illegible] = in-ner cortical zone OMZ = outer medullary zone IMZ = inner medullary zone

as distinct as in the fixed ones Tissue structure was poorly preserved, and the sections tended to disintegrate in the incubation medium The use of Millipore filters made slightly longer incubation times necessary, but the thin sections permitted better resolution

The *glomeruli* and *Bowman's capsule* were unstained

The cells of the *proximal tubules* in the outer cortical zone (= the convoluted part) were rather intensely stained, including the brush border, Fig 2, 3 (fixed) and 5 (unfixed) The proximal tubules in the inner cortical zone (= the straight part) were less intensely stained (not shown) The distribution of staining was not exactly the same as in the convoluted part—this matter is under further study

In the *thin segment of Henle's loop* both the nuclei and the thin cytoplasm were *distinctly stained in fixed sections*, Fig 6 *In unfixed sections however, no staining* was observed The reason for this is not clear at the present time

In the straight part of the *distal tubules* the whole cells were moderately stained, Fig 6 (fixed) 7 (unfixed) Thin sections showed a somewhat striated precipitation pattern clearly seen in the microscope In the convoluted part of the distal tubules the staining was restricted to the basal half or the basal thirds of the cells, Fig 3 (fixed) 5 (unfixed) and here too a striated precipitation pattern could be observed Unfixed sections showed a more intense staining than fixed sections in this part of the nephron

In the outer cortical zone another part of the kidney tubules showed very interesting staining caracteristics Some of the cells were stained only in the basal part and rather weakly so while other cells were heavily stained all over the cytoplasm, Fig 3 4 (fixed) 5 (unfixed) This part of the kidney tubules was identical with the *arched collecting tubules*, which was demonstrated when sections stained for carbonic anhydrase activity were matched with sections stained for mitochondria In these latter sections the arched collecting tubules were easily recognized as



Fig 2 Staining for carbonic anhydrase activity, no counterstain Outer cortical zone P = proximal tubule convoluted part Fixed 8μ thick Incubation time 6 min

Fig 3 Staining for carbonic anhydrase activity, no counterstain Outer cortical zone A = arched collecting tubule D = distal tubule, convoluted part P = proximal tubule, convoluted part Fixed, 4μ thick Incubation time 6 min

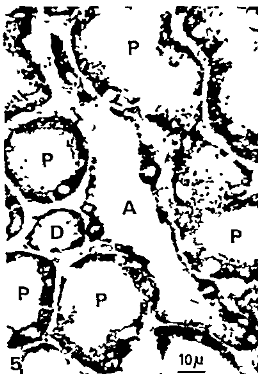
Fig 4 Staining for carbonic anhydrase activity, no counterstain Outer cortical zone A = arched collecting tubule Fixed 4μ thick Incubation time 9 min

are made up of two kinds of cells, one kind containing a great number of mitochondria the other kind containing rather few The *straight collecting tubules* in the inner cortical zone and the outer medullary zone were intensely stained In fixed tissue they were more intensely stained than any other part of nephron in these zones of the kidney, Fig 6 but in unfixed tissue the difference was much smaller Fig 7 The straight collecting tubules in the inner medullary zone were also stained, Fig 8, but towards the tip of the papilla the staining gradually became weaker Also in the straight collecting tubules weakly stained cells were found among the intensely stained ones, but they were more rare than in the arched collecting tubules

The nuclei in all parts of the kidney tubules were unstained when unfixed tissue was used, Fig 5, 7 In fixed tissue, however the nuclei were always stained except for the convoluted part of the distal tubules and the weakly stained cells in the collecting tubules This staining of the nuclei could be completely inhibited with 10^{-6} M acetazolamide, but its specificity has been questioned (Hansson 1967, 1968)

Discussion

Hausler (1958) was the first to demonstrate carbonic anhydrase activity in the proximal and distal tubules of the rat kidney but he could see no activity in the



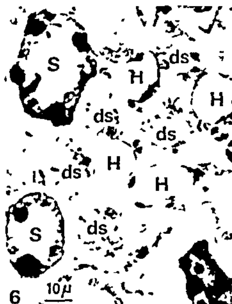


Fig 5 Staining for carbonic anhydrase activity, no counterstain Outer cortical zone A = arched collecting tubule D distal tubule convoluted part P – proximal tubule, convoluted part Unfixed, 4μ thick Incubation time 6 min

Fig 6 Staining for carbonic anhydrase activity no counterstain Outer medullary zone DS = distal tubule straight part H = thin segment of Henle s loop S – straight collecting tubule Fixed 8μ thick Incubation time 3 min

other parts of the kidney tubules Ishizaki (1969) using the histochemical method of Hausler (1958) and Waldeyer and Hausler (1959) reported that the staining reaction was restricted to the proximal and distal tubules also in the mouse kidney With his more sensitive method Hansson (1968) only investigated the proximal and *distal tubules in the outer cortical zone His findings of enzyme distribution in these* parts of the nephron were confirmed in the present study However, carbonic anhydrase activity could also be demonstrated in the thin segment of Henle s loop and in the collecting tubules This would correspond with the findings of Wistrand and Rao (1968) who reported immunological evidence for the presence of the enzyme in the cortical medullary and papillary regions of the dog kidney Using a modification of the indicator method of Roughton and Booth carbonic anhydrase activity has also been demonstrated in carefully dissected parts of the human kidney (Mattenheimer Pollak and Muehrcke 1970) They found activity in the inner and outer zone of the medulla, in the papilla and in isolated proximal and distal tubules from the cortex



7 Staining for carbonic anhydrase activity no counterstain Outer medullary zone DS = distal tubule straight part S = straight collecting tubule Unfixed 4μ thick Incubation time min

Fig 8 Staining for carbonic anhydrase activity no counterstain Inner medullary zone All stained tubular structures are straight collecting tubules E = erythrocytes The empty spaces are mainly occupied by vessels but unstained thin segments of Henle's loop may also be present Fixed 4μ thick Incubation time 6 min

The finding of carbonic anhydrase activity in the collecting tubules is in good agreement with physiological data Ullrich and Eigler (1958) reported that a large fall in pH occurred in the collecting tubules of hamsters Gottschalk Lassiter and Mylle (1960) using micropuncture techniques found that acidification in the rat kidney took place not only in proximal and distal tubules but also in the collecting tubules

From data obtained by micropuncture of the convoluted part of proximal and distal tubules of the rat kidney Rector Carter and Seldin (1965) concluded that luminal fluid is exposed to carbonic anhydrase activity in the proximal but not in the distal tubules These conclusions are consistent with the findings in the present study, as carbonic anhydrase activity was present in the brush border of the convoluted part of the proximal tubules whereas enzyme activity was restricted to the basal part of the cells in the convoluted part of the distal tubules

From many light and electron microscopic studies the arched collecting tubules are known to contain two kinds of cells the so called light and dark cells the latter also named 'Schaltzellen' or 'cellules intercalaires' (Rhodin 1958 Latta

Maunsbach and Osvaldo 1967) The dark cells contain a great number of mitochondria, while the light cells contain rather few Also the straight collecting tubules are made up of light and dark cells but the dark cells become less frequent and almost disappear towards the tip of the papilla

Hagege, Gabe and Richet (1968) have observed an increase in the number of dark cells (cellules intercalaires) when sodium or potassium bicarbonate was administered to rats The authors believed that this increase, which took place within 5 hours, was due to a functional transformation of the cells in this part of the kidney tubules, and postulated that the dark cells were involved in the tubular transport of bicarbonate ions The relation between light and dark cells as defined by conventional techniques, and the heavily and weakly stained cells found with the staining reaction for carbonic anhydrase activity is not clear but will be further studied

The distribution of enzyme activity along the kidney tubules as described in the present study, gives of course a wide variety of possible sites of action for inhibitors of the enzyme

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Efflux of Prostaglandins in Lymph from Scalded Tissue

By

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Abstract

ÄNGGÅRD, E and C-E JONSSON *Efflux of prostaglandins in lymph from scalded tissue* Acta physiol scand 1971 *81* 440—447

The smooth muscle stimulating lipids present in lymph from scalded dog paws were examined by a variety of chromatographic procedures in combination with bioassay It was shown that [illegible]

muscle stimulating activity appeared with the ^3H PGE$_2$ After treatment with alkali and con [illegible]

tion was observed It is concluded that most of the smooth muscle stimulating activity present in dog lymph after experimental burn injury is due to prostaglandin E$_2$

Smooth muscle stimulating lipids have recently been shown to appear in the lymph from dog paws following scalding by brief immersion in boiling water (Jonsson 1971) *In the present study this material has been further characterized by a variety* of chromatographic procedures as well as by enzymatic analysis The results show that most of the smooth muscle stimulating material can be identified as prostaglandin E$_2$

Material and methods

Collection of lymph A modification of the technique described previously was used (Arturson 1961) Vorsteh dogs of either sex weighing about 20 kg were anesthetized with sodium pentobarbital 30 mg/kg One or two lymphatic vessels from each of the four paws were cannulated [illegible] with a PE 50 polyethylene catheter [illegible]

after collection the fractions were transferred [illegible] an antioxidant, bishydroxy toluene (Wren and Szczepanowska 1964) or d l-α tocopherol The alcohol solution was stored at $-20°$ C until analyzed usually within one week

LYMPH + ETHANOL + ^{3}H PGE_2
↓ filtration, evaporation
CRUDE LYMPH EXTRACT
pH 3 ↓ → HEPTANE
ETHER
pH 8 ↓
PHOSPHATE BUFFER
pH 3 ↓
ETHER
↓ evaporation
ACIDIC LIPID EXTRACT

Fig 1 Flow diagram showing extraction of smooth muscle stimulating material from lymph

Extraction of the smooth muscle stimulating activity A flow diagram of the extraction procedure is shown in Fig 1 A known small amount (0 15 μC, 10 ng) of ^{3}H labelled prostaglandin E_2 (PGE_2) (Änggård, Gréen and Samuelsson 1965) with a specific activity of 4 5 C/mmole was added to the alcoholic lymph After filtration the clear filtrate was evaporated under reduced pressure at room temperature to a small volume This extract was designated "crude lymph extract" (Fig 1) After extraction with two volumes of n heptane, which were discarded the aqueous phase

twice with two volumes of eth

two volumes of 0 1 M potass

HCl and extracted twice witl

small amounts of water the e

Reversed phase partition chromatography was performed on 4 5 g of hydrophobic Supercel using 4 ml of iso-octyl alcohol chloroform (1 1) as the stationary phase and 300 ml of

After extraction into ether at pH 3 the material was treated with diazomethane The methyl

was used This instrument was equipped with a ^{63}Ni electron capture detector operated with a pulse generator Pulse interval 50 μsec Detector temp 270° C Column 1 % OV 1 on 100—110 Gas-chrom Q temp 225° C carrier gas 10 % methane in argon

Enzymatic identification Biologically active material isolated from lymph by extraction and silicic acid chromatography was used Incubation of smooth muscle stimulating material corresponding to 24 ng of PGE_2 was carried out at 37° for 30 min in 0 2 ml 0 1 M Tris buffer pH 8 0, containing 5 mM NAD^+, 0 15 mU of 15 hydroxy prostaglandin dehydrogenase isolated

from swine lung (Änggård and Samuelsson 1966, 1970, Nakano, Änggård and Samuelsson 1969) Control incubations of the same material were performed where NAD⁺, prostaglandin dehydrogenase or both were omitted from the incubation medium Directly after the incubation period an aliquot of the sample was added to the smooth muscle bath for biological assay as described below

Biological assay The biological activity in the extracts and in the chromatographies was assayed on the rat fundus strip according to Weeks Schultz and Brown (1968) A 1 5 cm strip was mounted in a 1 ml glass bath oxygenated and kept at 37° The preparation was suspended in Tyrode solution and connected to an isotonic transducer (Harvard Apparatus Cambridge, Mass) Contractions were recorded for 90 sec Total cycle time was 8—10 min Usually a 3 point assay technique was employed Material present in organic solvent was reduced to dryness by a stream of nitrogen and dissolved in Tyrode solution before assay

Results

Recovery of smooth muscle stimulating activity in the acidic lipid extract In 3 expts the smooth muscle stimulating activity was tested in the crude lymph extract and in the acidic lipid extract The recovery of biological activity in the acidic lipids was found to be 98, 85 and 94 % respectively of that in the crude lymph extract after correction for losses through the extraction procedure Thus nearly all of the smooth muscle stimulating material present in the lymph appears to be of acidic lipid character

Chromatography of smooth muscle stimulating lipids The material present in the acidic extract was first subjected to silicic acid chromatography This system achieves ...ip separation of the prostaglandins (Samuelsson 1963) The result is shown in Fig 2 It is seen that a peak representing the major part of the total biological activity appears concomitantly with the radioactivity due to the internal standard of

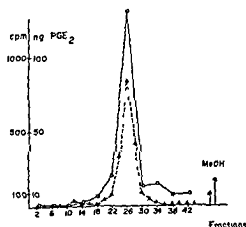


Fig 2

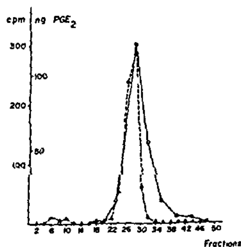


Fig 3

Fig 2 Silicic acid chromatography of smooth muscle stimulating compounds from lymph Silicic acid 1 g Linear gradient of ethyl acetate benzene in volumetric ratios from 1 9 to 9 1 Fractions 1 5 ml Smooth muscle assay o——o radioactivity ▲– – –▲

Fig 3 Reversed phase partition chromatography of material isolated from lymph by extraction and silicic acid chromatography Fractions 3 ml Bioassay o——o, radioactivity ▲– – –▲

³H PGE₂ No smooth muscle stimulating compounds appeared earlier in the chromatography and only trace amounts in the methanol eluate This pattern was found to be present in two out of three silicic acid chromatographies In one chromatography about 30 % of the biological activity appeared after the main peak at the expected position of the prostaglandin F-compounds

The material isolated from lymph by extraction and silicic acid chromatography was subjected to reversed phase partition chromatography as described under Methods This procedure affords separation of the individual prostaglandin E compounds (Bergstrom *et al* 1962) The smooth muscle stimulating material appeared in a single peak coinciding with that due to the labelled PGE_2 The chromatography is shown in Fig 3 Small amounts of biological activity was also observed to appear after the radioactive peak at the expected position of PGE_1

Biologically active material isolated from lymph by extraction and by silicic acid and reversed phase partition chromatography was analyzed by gas liquid chromatography with electron capture detection After conversion to the PGB-compounds by treatment with alkali and conversion to the methyl ester with diazomethane the material was further purified by chromatography on Sephadex LH 20 The radioactive material appearing in the effluent was converted to the trimethylsilyl ether and injected into the gas chromatograph The chromatogram is seen in Fig 4 A large peak having the same retention time as the corresponding derivative of authentic PGB was observed The amount of PGE_2 in the sample calculated by gas chromatographic measurements corresponded closely to that obtained by biological assay of the original material

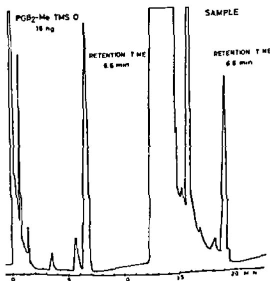


Fig 4 Gas chromatography of material isolated from lymph The reference PGE_2 and the sample were treated with alkali and resulting PGB-compound converted to the methyl ester trimethylsilyl ether (Me TMSiO) Conditions 1 % OV 1 temp 225° ^{63}Ni electron capture detector

TABLE I Inactivation of smooth muscle stimulating compounds with 15 hydroxy prostaglandin dehydrogenase (PGDH)

LYMPH EXTRACT	ADDITIONS PGDH (0 15 mU)	NAD+ (5 mM)	BIOASSAY (ng of PGE_2)
+	+	+	0
+	—	+	24
+	+	—	20
+	—	—	24
—	+	+	0

Enzymatic identification 15 Hydroxy prostaglandin dehydrogenase isolated from swine lung is specific for the 15 (S) hydroxy group in the prostaglandins (Änggård and Samuelsson 1966 Nakano Änggård and Samuelsson 1969 Shio *et al* 1970) The resulting 15 keto metabolites possess only a few percent or less of the biological activity of the parent compound (Änggård 1966 Pike Kupiecki and Weeks 1967) When the smooth muscle stimulating material isolated from lymph was incubated with prostaglandin dehydrogenase and NAD complete biological activation was observed (Table I) Control incubations omitting either the enzyme or the cofactor retained full activity It appears therefore that the smooth muscle stimulating compound(s) present in the lymph is a substrate for the 15 hydroxy prostaglandin dehydrogenase This finding then constitutes further strong evidence as to the prostaglandin nature of the spasmogenic substance(s) appearing in the lymph after burn injury

Discussion

Release of histamine kinin forming activity and small amounts of serotonin in peripheral dog lymph following burn injury was previously reported by Edery and Lewis (1963) In our experiments we used the rat fundus strip which is sensitive to prostaglandins and serotonin but not to histamine and bradykinin (Vane 1957 Weeks Schultz and Brown 1968) The fact that most of the smooth muscle stimulating activity present in the crude lymph extract could be accounted for by prostaglandins does therefore not exclude the presence of histamine and certain peptides

The prostaglandins were found to be mostly of the E type Little biological activity was found at the expected position of the prostaglandin F compounds in the silicic acid chromatographies It should be recalled however that the rat fundus strip is more sensitive to prostaglandin E as compared to prostaglandin $F_{2\alpha}$ (Weeks Schultz and Brown 1968) Since nearly all activity obtained from the silicic acid chromatography appeared together with the labelled prostaglandin E in the reversed phase system it is concluded that most of the biological activity was in fact due to this compound

Previous investigations have indicated that a variety of procedures stimulate the formation and release of prostaglandins Thus nerve stimulation (Ramwell and Shaw 1963, 1966, Coceani and Wolfe 1965, Ramwell Shaw and Kucharski 1965 Coceani *et al* 1967, Laity 1969) venoms (Vogt *et al* 1969, Ladinsky and Strandberg 1969) anaphylaxis (Änggård *et al* 1963, Piper and Vane 1969), histamine releasers (Laity and Moore 1970), ischemia (Edwards, Strong and Hunt 1969 McGiff *et al* 1969) mechanical stimulation (Edmonds Berry and Wyllie 1969 Gilmore Vane and Wyllie 1969) and osmotic shock (Vogt and Distelkotter 1967) release prostaglandins It appears that alterations in membrane function is the only feature common to all these situations It is therefore not surprising that release of prostaglandins can be demonstrated also following burn injury The mechanism by which membrane damage activates cellular prostaglandin biosynthesis is however not known

A variety of biologically active substances have been proposed as mediators of the inflammatory response to trauma, drugs and toxins (Spector and Willoughby 1968) Our results show that in burn injury prostaglandins must also be included as possible humoral mediators Interestingly a prostaglandin like activity has been reported to occur during carrageenin induced inflammation in the rat (Willis 1969) Since prostaglandin E-compounds are powerful capillary dilatators and induce increased vascular permeability in the skin (Solomon Juhlin and Kirschenbaum 1967 Juhlin and Michaelsson 1969, Viguera and Sunahara 1969, Weiner and Kaley 1969 Crunkhorn and Willis 1970) it seems possible that prostaglandins released in the extracellular fluid could contribute to local as well as systemic manifestations of the burn injury syndrome

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The Effect of Sympathetic Nerve Stimulation on Pulmonary Blood Volume in Isolated Perfused Lungs

By

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Abstract

AARSETH, P G NICOLAYSEN, and B A WAALER *The effect of sympathetic nerve stimulation on pulmonary blood volume in isolated perfused lungs* Acta physiol scand 1971 *81* 448—454

Stimulations of the vago-sympathetic nerve trunk at 5 10 imp/sec have been carried out in isolated dog lung preparations perfused at constant volume inflow When the left atrial pressure was kept at 3—4 cm of water the predominant response to these stimulations was a weight reduction of the preparation This response is interpreted as being due to a reduction pulmonary blood volume Nerve stimulations caused only small weight reductions or even weight augmentations, when the left atrial pressure was at or near zero The blood volume reductions resulting from nerve stimulations appeared to be mediated via sympathetic nerve fibres and an a receptor mechanism

Various authors have described that considerable changes in pulmonary blood volume might occur with different circulatory conditions such as *e g* exercise hypervolaemia and acute hemorrhage (Korsgren *et al* 1969, De Pasquale Hyman and Burch 1965, Aarseth 1970) The large volume of this vascular bed and also its position upstreams to the left heart ventricle calls for more information about the magnitude and pattern of its blood volume alterations In this context one would like to know to what extent humoral and/or nervous stimuli might influence or control the pulmonary blood volume It has been shown that injections of catecholamines cause reductions in the blood volume of isolated perfused lungs (Daly Foggie and Hebb 1940 Hauge Lunde and Waaler 1967) In lungs of rabbit and cat this effect was shown to be mediated via an *a* receptor mechanism and to be dissociated from the resistance effects of the catecholamines on the pulmonary vascular bed (Hauge *et al* 1967) From experiments on isolated lungs there is also some information on the influence of nervous stimuli on pulmonary blood volume In isolated lungs where the capillaries were completely blocked by high intratracheal pressure, Daly and Waaler (1961 b) thus observed that stimulation of the vago sympathetic nerve trunk caused some blood volume reduction on the arterial as well as on the venous side of the

capillaries In their recent analysis of the site of action of nerves in the pulmonary vascular bed Daly, Ramsay and Waaler (1970) also found a lung blood v... e reduction in isolated preparations, reflected by a temporary increase in fl... flow, when the vago-sympathetic nerve trunk was stimulated at 47 imp ...

The intention with the present investigation was to study more closely the and magnitude of lung blood volume changes occurring on stimulation of th... sympathetic nerve trunk, particularly at stimulation frequencies of 5—10 imp... Attention was also paid to the role played by the level of the left atrial and p... monary venou pressure for the lung blood volume alterations caused by nerve sti... lation From systemic veins it is known that the level of the transmural vascular pressure is important for the magnitude of volume changes induced by stimulation of sympathetic nerves (Öberg 1967) An isolated perfused dog lung preparation was used for the present experiments, and it could be shown that a considerable blood volume reduction was the predominant vascular response to appropriate nerve stimulations

Methods

Animals Dogs weighing between 11 and 20 kg were used They were premedicated with morphia 1 mg/kg b w i m The femoral artery was then disected free and cannulated under ... ned The chest was opened with a mid ... quently the lobes of the right lung and ... necessary dissection was carried out and ... The preparation consisted of the two upper left lung lobes with their vascular supplies (including the bronchial vascular supply) the heart with the caval veins tied off—but with the aorta and pulmonary vessels open the left vago-sympathetic nerve up to the stellate ganglion and a small part of the oesophagus (closed by ligatures at both ends) It was attempted to keep the blood supply to the nerves intact by keeping a long part of the left subclavian artery (which was tied off at the level of the ganglion) with the preparation Some small arterial branches from it to the nearby nerve was thus preserved The aorta was tied off below the 5th or 6th pair of intercostal arteries Great care was taken to preserve the bronchial arterial supply through one big bronchial arterial branch ... All other arteries branchir...

Perfu... ... described by Daly and Wa... pulmonary vascular bed of the lung preparation was perfused with a Dale and Schuster pump through a cannula positioned in the pulmonary artery The blood was drained back to a reservoir from a cannula in the left auricle The bronchial vascular system was perfused with another Dale and Schuster pump through the cannulated thoracic aorta.

The preparation was to some extent directly suspended via a string tied around the non beating and non perfused heart At the same time the lung lobes were supported on a perforated plastic plate—which again via strings from its corners—togther with the string from the heart—was suspended underneath a FTA 100 1 Sanborn force transducer (Fig 1) This transducer was connected to a Sanborn 350—1100 C carrier preamplifier and further to a ... by a suitable ... the prepara... displacement ...

Pulmonary arterial pressure (P A p) was recorded from a s de branch of the cannula in the pulmonary artery with a Statham P23Db transducer connected via another Sanborn 350—1100 C carrier preamplifier to the Sanborn dual channel recorder

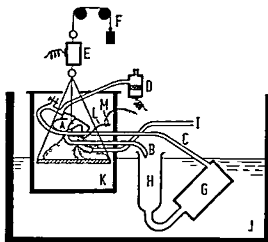


Fig 1 *Schematic drawing of the perfusion and recording arrangement for the isolated lung preparation with its intact nerve supply* The two lung lobes are suppled A the non beating heart B perfusion outflow cannula (from the left auricle) C perfusion inflow cannula (into pulmonary artery) D pressure transducer for recording of pulmonary arterial pressure E force transducer for recording of changes in preparation weight F counter balance system G perfusion pump H perfusate reservoir I cannula for ventilation J thermostatically controlled waterbath K organ chamber L the vago-sympathetic trunk M electrode for nerve stimulation

The whole preparation was placed inside a plastic casing which—together with the blood reservoir and pump—was thermostated at 37° C

Perfusate Autologous heparinized whole blood was used as a perfusate Before exsanguination the animals were given about 3—4 mg/kg of dissolved pure powdered heparin (Novo) (130 I U /mg) i v About twice this amount of heparin (8—9 mg/100 ml blood) was placed in the cylinder into which the animal was exsanguinated The final heparin concentration of the blood was thus about 1500 I U /100 ml The blood was kept at 37° C until used for perfusion

In some cases also 1 mg of atropine was given intravenously before exsanguination

Ventilation Positive pressure ventilation with a Starling Ideal pump (C F Palmer Ltd London) was carried out Maximal inflation pressure and expiratory pressure were kept at 10 and 2 cm of water respectively

Nerve stimulation The left vago-sympathetic nerve was placed on a bipolar platinum electrode and stimulated with square wave impulses from a Palmer electronic square wave stimulator

As an *inhibitor* of α-effects of catecholamines phentolamine methansulphonate (Regitin® Ciba) was used

Results

The three last experiments performed were completely successful in that the preparation could be perfused and effective nerve stimulations performed for hours without any development of edema Some initial experiments were less successful in that there developed edema at a stage where none or only a few nerve stimulations had been carried out It appeared that the reason for this edema development was connected to filtration of the blood through gauze a procedure which was therefore abandoned

The results presented are those from the three completely successful experiments Results from three other experiments in which 3—4 nerve stimulations could be performed before edema developed were however very similar to those obtained in the best experiments

In each of the three successfull experiments a series of nerve stimulations at 5 or 10 imp/sec (each impulse lasting 2 or 5 msec) were carried out For each experi

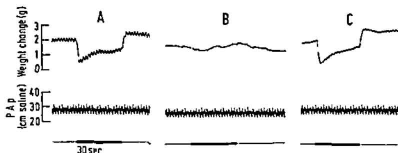


Fig 2 *Alterations in pulmonary weight and in pulmonary arterial pressure of an isolated perfused dog lung preparation (Experiment 1) on stimulation of vago sympathetic nerve* Preparation two upper left lung lobes from 14 kg dog Constant volume inflow perfusion For other details with perfusion arrangement see text At signals stimulations for 30 sec of left vagosympathetic nerve Stimulation parameters 10 V 2 msec, 10 imp/sec Upper tracing weight alterations of preparation upward deflection = increase in weight downward deflection = decrease in weight Lower tracing pulmonary arterial pressure (P A p) in cm of water

A Stimulations carried out at L A p level of 3 5 cm of water
B Stimulations carried out at L A p level of 0 5 cm of water
C Stimulations carried out at L A p level of 6 5 cm of water

ment the stimulus strength was increased until one got a maximal weight response This occurred at voltages varying from 10 to 20 V In some experiments the stimulations had bronchomotor effects, which could conceivably affect vascular volume However, when the bronchomotor effect of stimulation had been abolished by added atropine (1 mg), the weight responses to stimulation of the vago sympathetic nerve were unchanged

When left atrial pressure (L A p) was kept above 1 cm of water nerve stimulation caused a marked weight reduction occurring within a few sec (Fig 2) or more slowly and then reaching its maximum within 20—25 sec (Fig 3) Although the stimulation was carried on the weight then slowly increased This increase which seemed to be more marked the larger the initial weight reduction had been continued after the stimulation had ceased (Fig 3) The maximal weight reduction obtained was 1 3 g in one preparation and 1 6 g in the two others

When L A p was kept at 0—0 5 cm of water the initial weight reduction during vago sympathetic nerve stimulation was small or absent (Figs 2 B and 3 B)

There was hardly any weight response to nerve stimulations when both pulmonary and bronchial vascular perfusions were stopped and when at the same time in as well as outflow cannulas were closed (Fig 3 E)

The response of weight reduction to nerve stimulation was gradually abolished after the addition of 3 mg Regitin®/100 ml of blood (Fig 3 G)

The type of stimulations applied caused only small or moderate increases in mean pulmonary arterial pressure (P A p) and in pulse pressure The increase in pulse pressure was usually more apparent than that in mean P A p

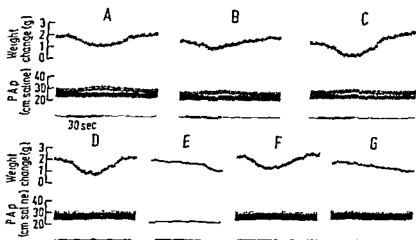


Fig 3 Alterations in pulmonary weight and in pulmonary arterial pressure of an isolated perfused lung preparation (Experiment 4) on stimulation of vago sympathetic nerve Preparation two upper left lung lobes from 12 kg dog Constant volume inflow perfusion For other details with perfusion arrangement see text At signals stimulation for 30 sec of left vago-sympathetic nerve Stimulation parameters 17 V 5 msec 5 imp/sec Upper and lower tracings as in Fig 2

A C D F and G Stimulations carried out at a left atrial pressure (L.A p) level of 4 cm of water B Stimulation carried out at L A p level of 0 cm of water At E Stimulation carried out with perfusion stopped and with aorta pulmonary artery and outflow cannula closed F and G—and about 4 min before stimulation at G 10 mg of phentolamine methanol sulphonate (Regitin®) added to perfusate reservoir

Discussion

Stimulations of the vago sympathetic nerve trunk have been shown to cause relatively marked alterations in preparation weight in the present series of experiments *These weight alterations occurred at a stimulation frequency of 5—10 imp/sec* which should be within the range of normal impulse frequencies for autonomic nerve fibres The predominant response seen was a weight fall which began within 1 sec after start of stimulation and its maximum was reached within from 3 to 25 sec of stimulation. The question then arises as to what sort of alterations within the preparation is causing the observed fall in weight There is hardly any other part of the preparation than the two lung lobes which could alter its weight to such an extent. The heart was not perfused The oesophagus was closed at both ends and the reduced aorta could hardly have altered its volume by as much as 1 6 ml Nor could a weight reduction of this dimension be due to constriction of bronchial vessels There is no indication that the capillary pressure was markedly reduced causing an inward transvascular flux of fluid with interstitial tissue weight reduction as its consequence The reduction in lung weight must therefore mainly reflect a reduction in pulmonary vascular volume This conclusion which is in agreement with the findings of Daly *et al* (1970) is strongly supported by the observation that no weight fall occurred when during nerve stimulation the pulmonary vascular bed was clamped off at both ends (Fig 3 E)

It has been shown that systemic veins must be somewhat distended for a measurable volume reduction to appear on sympathetic nerve stimulation (Öberg 1967). In agreement with this we obtained marked lung weight reductions on nerve stimulations only when the L.A.p. was kept above a certain level. The dependence of the weight reduction response on an outflow pressure somewhat above zero seems to indicate that the observed reduction in vascular volume is largely occurring in veins.

There is reason to conclude therefore that stimulation of the vago-sympathetic nerve trunk at reasonable stimulation frequencies can give rise to marked reductions in pulmonary blood volume. This effect appears to be mediated via adrenergic nerve fibres and an α-receptor mechanism, since it was abolished by Regitin®.

Even if these observations were done on an isolated organ preparation under highly artificial conditions, the findings do nevertheless support the notion that pulmonary blood volume can be reduced as a result of activity in the sympathetic nerve fibres to the lung vessels.

The size of the response—a 1.3—1.6 g weight reduction of the two upper left lobes—is not a small one. A 12—14 kg dog would have a blood volume of about 1000 ml. The pulmonary blood volume would be perhaps 100 ml. The fraction of this present in the two perfused lobes is of the order of 15—20 ml. The reduction in blood volume obtained is thus of the order of 5—10 %.

That weight increases occurred during stimulations, both at low venous pressures —and subsequent to the initial weight fall seen at left atrial pressure levels of 3—4 cm of water, might well be explained as secondary to a vasoconstrictor response. A passive distension of vessels upstreams to constricted venous vessels could give passive vascular distension as well as some net outward filtration of fluid from small vessels. The time course of weight increases indicate that such mechanisms might be operating. The maximal weight increase was thus seen after stimulation had been ended and it took up to several min to decline completely.

The nerve stimulations did not to any great extent affect the mean P.A.p., the pulse pressure did, however, usually show some increase. The picture as far as pressure alterations are concerned, was thus more that of a reduction in vascular compliance. This finding is in agreement with the observations of Szidon and Fishman (1969). In previous experiments with pulmonary vasomotor nerve stimulation in isolated lungs much more marked alterations in total vascular resistance have been achieved (*e.g.* Allison, Daly and Waaler 1961). In those experiments, however, much higher stimulation frequencies (about 50/sec) were used. With the stimulation frequency chosen in the present experiments, alterations in preparation weight were a more striking part of the response than alterations in pulmonary vascular resistance.

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Acta physiol. scand. 1971. 81. 455—458

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Pyruvate and Lactate Ratios in Muscle Tissue and Blood during Exercise in Man

By

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Abstract

KARLSSON, J. *Pyruvate and lactate ratios in muscle tissue and blood during exercise in man.* Acta physiol. scand. 1971. *81.* 455—458.

To study the changes by exercise in the relationships betwen pyruvate (Py) and lactate (La) in muscle tissue and blood of man, Py and La were determined in muscle biopsy specimens (M. quadriceps femoris) and in blood in 8 subjects at rest (I) and after submaximal (II) and maximal (III) bicycle exercise. In the resting muscle Py was 0.06 mmoles $\times$ kg^{-1} wet muscle and increased with exercise to 0.14 (II) and 0.13 (III) mmoles $\times$ kg^{-1} whereas La increased from 2.3 (I) to 10.4 (II) and 17.1 (III) mmoles $\times$ kg^{-1} wet muscle. In the blood the corresponding values for Py were 0.11 (I), 0.24 (II), and 0.39 (III) mmoles $\times$ l^{-1} and for La 1.6 (I), 7.7 (II) and 10.0 (III) mmoles $\times$ l^{-1}. Thus the La/Py ratio increased from 38 (I) to 74 (II) and 131 (III) in the muscle and from 15 (I) to 32 (II) and 26 (III) in the blood. The increase in the muscle Py concentration during exercise could not account for more than a very minor part of the muscle La concentration which means that the excess lactate (XL) is of very minor physiological importance.

Pyruvate and lactate are interconvertible in the presence of the enzyme lactate-dehydrogenase (LDH) and excess of nicotinamide-adenine dinucleotide (NAD) in its reduced or oxidized form. In general the equilibrium is supposed to be defined by the mass action law, the pH, the properties of the enzyme, and to what extent extramitochondrial NADH is present. According to the concept of the mass action law, lactate might be expected to be formed due to an accumulation of pyruvate in the cell. On this basis the term "excess lactate" (XL) was introduced by Huckabee in 1958, calculated from simultaneous lactate and pyruvate determinations in blood. According to the concept XL would better correlate to the oxygen deficit and the degree of anaerobiosis than the lactate concentration per se.

The present study was then performed to simultaneously determine pyruvate and lactate concentrations at rest and during exercise in blood and the exercising muscle and to evaluate whether a pyruvate accumulation was present in the muscle as earlier described for lactate (Diamant, Karlsson and Saltin 1968).

Subjects

8 healthy physical education students with a mean age of 23 (range 21—24) years participated in the study Their mean height was 178 cm and mean weight was 70 kg Maximal oxygen uptake averaged 56 (range 51—66) ml × kg ¹ × min ¹.

Methods and procedure

Concentrations of lactate and pyruvate were determined enzymatically in blood (Scholz *et al* 1959) and muscle tissue (Lowry *et al* 1964, and Karlsson, Diamant and Saltin 1970)

portion of the thigh (M *quadriceps femoris*) *at rest with the subject in supine position and immediately after exercise sitting on the bicycle All samples were frozen within 3—4 sec after cessation of work* Simultaneously, blood samples were drawn from a prewarmed finger tip to determine blood lactate and pyruvate concentrations

Results

Mean values are given in Table I All individual values for muscle pyruvate and tate concentrations are shown in Fig 1

At rest the mean muscle pyruvate (Py) concentration was 0 06 mmoles × kg ¹ and increased during submaximal and maximal exercise to 0 14 and 0 13 mmoles × kg ¹, respectively In the corresponding situations muscle lactate concentration (La) was 2 3 10 4, and 17 1 mmoles × kg ¹ Thus the La/Py in the muscle tissue being 38 at rest was increased to 74 during submaximal and to 131 during maximal exercise In the blood the La/Py ratio was 15 at rest Lactate concentration was 1 6 and pyruvate concentration 0 11 mmoles × l ¹ The corresponding ratios at submaximal exercise and at maximal exercise were 32 and 26 respectively, lactate concentration being 7 7 and 10 0 and pyruvate concentration 0 24 and 0 39 mmoles × l ¹

TABLE I Mean values and range are given for the work loads and oxygen uptakes Muscle and blood concentrations of pyruvate and lactate, respectively, are presented as means ± SE and SD

	Work load	Oxygen uptake	Pyruvate		Lactate	
	kpm min ¹	l × min ¹	muscle*	blood**	muscle*	blood**
Rest n = 8	—	—	0 06 ± 0 01 0 03	0 11 ± 0 02 0 7	2 3 ± 0 2 0 7	1 6 ± 0 1 0 3
Submaximal work n = 5	1290 (1200-1350)	3 1 (2 8 –3 2	0 14 ± 0 02 0 04	0 24 ± 0 03 0 06	10 4 ± 0 6 1 3	7 7 ± 0 3 1 1
Maximal work n = 5	2010 (1950—2100)	3 9 (3 8—4 1)	0 13 ± 0 03 0 07	0 39 ± 0 05 0 11	17.1 ± 1 6 3 5	10 0 ± 1 1 2 4

* mmoles × kg⁻¹ wet muscle
** mmoles × l⁻¹ blood

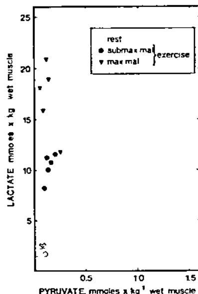


Fig 1 Muscle lactate concentrations are presented in relation to muscle pyruvate concentrations at rest and immediately after a submaximal and maximal work load

Discussion

The resting pyruvate concentration in the blood was higher than that in the muscle tissue which is also true if the comparison is made on per liter of water basis The water content has been found to range 76—79 per cent of the wet weight in a material of biopsy specimens obtained and treated similarly to the present (unpublished results) This discrepancy in pyruvate concentration between muscle and blood tended to be enhanced during exercise It seems therefore reasonable to assume a concentration gradient of pyruvate from the extracellular to the intracellular space Since the gradient is small and the pH of the extra and intracellular spaces may favour the observed distribution an uptake of pyruvate by the muscle may not be present (Siesjo Granholm and Kjallquist 1968)

At rest in a canine gracilis muscle a negative a v difference for pyruvate has been observed (Karlsson Rosell and Saltin 1971) During stimulation that caused the muscle lactate concentration to increase to approximately 17 mmoles$\times$kg^{-1} wet muscle and lactate to be released to the venous blood in the order of 0 2—0 3 *mmoles$\times$kg$^{-1}\times$min^{-1} a positive a v difference for pyruvate was observed* This can be explained either by an uptake of pyruvate by the muscle or a reduction of extracellular pyruvate by membrane located LDH fractions The latter possibility has been suggested by Siesjo Granholm and Kjallquist (1968) to be valid for brain tissue

The present data do not confirm Huckabee s basic assumption that blood lactate and pyruvate concentrations reflected intramuscular concentrations On the other hand there was an increased pyruvate concentration in the muscle during exercise as postulated by Huckabee but its quantitative role for the observed lactate accumu

lation must be minimal (*cf* Fig 1) Recent studies indicate that at least during very heavy exercise the accumulation of lactate is directly related to the oxygen deficit (Karlsson and Saltin 1970) in the exercising muscles

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Acta physiol. scand. 1971. 81. 459—471

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Loci of Neurogenic and Metabolic Effects on Precapillary Vessels of Skeletal Muscle

By

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Abstract

Folkow, B, R. R Sonnenschein, and D L Wright *Loci of neurogenic and metabolic effects on precapillary vessels of skeletal muscle* Acta physiol scand 1971 *81* 459—471

By cannulation of a branch of the proximally clamped sural artery of the anesthetized cat distal arterial pressure (DAP) in the gastrocnemius muscle was recorded Measurement of blood flow femoral arterial pressure and DAP allowed calculation of total resistance (R_T) included precapillary sphincters and the hich included the larger arteries With was accounted for mainly by constric x, progressive constriction of proximal vessels accounted for most of the elevated R_T during the steady state subsequent reactive hyperemia mainly involved distal vessels R_T was less affected by sympathetic stimulation during exercise than when the muscle was at rest constriction of distal vessels was more markedly reduced than that of proximal vessels Ascending dilatation was evident during exercise Sympathetic cholinergic vasodilatation mainly involved vessels more proximal to those which were dilated early in exercise The findings are compatible with the concept that capillary flow distribution as a function of terminal arterioles and precapillary sphincters is adjusted by local factors towards an optimum for the prevailing metabolic level of the tissue

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Recent investigations have indicated that the components of the peripheral vasculature even in a single organ, may behave quite differently, one from another, in response to various stimuli Some years ago for instance Zweifach (1961) summarized findings from direct observation chiefly on the microvessels of the rat mesentery pointing out a gradient of increasing sensitivity to humoral agents from arteries to precapillary sphincters accompanied by a gradient of decreasing responses to vasoconstrictor fiber activation

Indirect measurements have confirmed the differential responsiveness of the sequential vascular segments to particular stimuli For instance, Cobbold *et al* (1963) measured blood flow and capillary filtration coefficient (CFC) of resting skeletal muscle of the cat during sympathetic vasoconstrictor stimulation, and ob-

served an initial reduction in CFC which, with continued stimulation, rose to or above its initial value even while total resistance remained elevated Thus while the resistance vessels remained constricted, apparently the precapillary sphincters rapidly relaxed Similar observations by Kjellmer (1965) of flow and CFC in exercising muscle during sympathetic stimulation also pointed to the relatively independent behavior of those vascular segments involved in control of resistance and those responsible for regulating the number of open capillaries

Since Haddy's (1954) description of the measurement of 'small vessel' pressures he and others among them Abboud and Eckstein (1966) and Zimmerman (1966) have exploited this method in investigating the selective effects of physiological and pharmacological procedures on various segments of the peripheral vasculature

In the present experiments we have adapted Haddy's technique to help elucidate the differential vascular responses in skeletal muscle to certain physiological stimuli The results some of which have been presented in a preliminary form (Folkow *et al* 1968) bear on the following questions What are the temporal aspects of sympathetic vasoconstriction and exercise dilatation at different arterial sites? At what locus does interaction take place between sympathetic vasoconstriction and exercise dilatation? What is the site of action of the sympathetic cholinergic vasodilator innervation and how does this compare with exercise dilatation?

Methods

Cats of either sex 32 in number weighing 2 0 to 3 5 kg were in general anesthetized with pentobarbital sodium 30 mg per kg intraperitoneally supplemented by additional intravenous doses as required In the few cases in which i v chloralose 30—40 mg per kg (supplemented by urethane as needed) was used results were as with pentobarbital Body temperature measured with an intraesophageal thermistor was maintained at approximately 37° C with an external heat lamp The trachea was cannulated and the left femoral artery and vein cannulated for respectively arterial pressure measurement and i v injections

The skin of the right leg was slit longitudinally and removed from the underlying tissue after the leg was fully prepared the skin was replaced and muscle temperature was maintained at 34—36° C with a heat lamp The ankle was tied off tightly to eliminate its blood flow The right femoral artery was gently dissected free and its major branches in the thigh tied off A ... the artery

going to the calf and ... ments noted in the text arterial flow was maintained constant by use of a Sigmamotor peristaltic pump acting on a polyvinyl cannula which directed all flow to the femoral artery from a carotid or the opposite femoral artery

... the gastrocnemius muscle were dissected ... of the artery and its accompanying vein ... was inserted into this arterial branch in ... ry pressure When the sural artery was ... essure (DAP) was then recorded (In a ...

monitored ... same type or a P23A transducer Outputs from the pressure transducers and ... flowmeter were recorded on a Grass polygraph

The right sympathetic chain from the L4 to the L6 ganglia was exposed and a bipolar silver or stainless steel electrode was placed on the L4—L5 or L5—L6 segment the nerve was cut

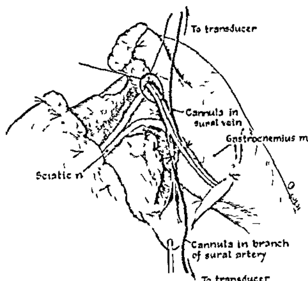


Fig 1 Lateral view of the hind leg of the cat showing the location of the cannula in a branch of the sural artery for recording of small artery pressure, and site at which the sural artery was occluded (indicated by the knotted thread) to obtain "distal arterial pressure" (DAP) Pressure in the sural vein was not recorded in all experiments

centrally The chain was stimulated at just supramaximal voltage with square wave pulses of 4 msec duration, at frequencies ranging from 0 5 to 8 Hz For inducing contractions of the calf muscles a similar electrode was placed on the sciatic nerve just central to its division into the tibial and common peroneal nerves Stimulation at 1 or 2 sec intervals was with 40—100 msec [illegible]

resistance The relative contribution of distal vessels to the total resistance at any moment was indicated by the ratio R_D/R_T at that moment To evaluate the extent to which distal vessels contributed to a change in resistance from the control state calculation was made of the ratio $[(R_D/R_T)_2/(R_D/R_T)_1] = q$ where $(R_D/R_T)_2$ was the relationship at a given moment during the experimental procedure and $(R_D/R_T)_1$ was that during the control period Thus for example, during vasoconstriction a value of $q>1$ indicated that distal vessels were constricted relatively more than proximal vessels, conversely, during vasodilatation if $q<1$ the distal vessels were dilated relatively more, etc It is important to keep in mind that this approach can give valid information only on the direction and time course of changes in the proximal and distal segments, and not on absolute values of these changes

Results

1 *General observations*

The mean small artery pressure (SAP) before the sural artery was clamped proximally was generally 95—98 per cent of the mean femoral arterial pressure (FAP) Pulsations were moderately forceful, although damped with respect to those in the femoral artery

When the sural artery was clamped, the distal small artery pressure (DAP) fell immediately reaching a low point in 10—15 sec, and then gradually rose to attain a steady level in about 1 min This level varied widely among the experimental animals from 12 5 to 85 mm Hg, corresponding to 11 to 70 per cent of the mean femoral pressure (FAP), two-thirds of the cases were between 25 and 55 per cent

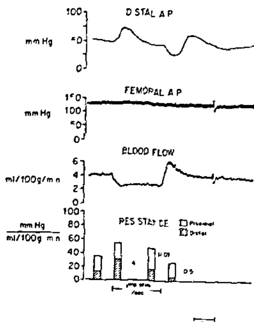


Fig 2 Vasoconstrictor response to stimulation of the sympathetic chain From above downwards the distal and damped femoral arterial pressures (note differences in scales) femoral arterial flow and calculated resistances during control at the initial peak rise in DAP during the steady state and at the peak of reactive hyperemia occurring after cessation of stimulation The numbers in parentheses following the bars represent ing the resistances are the respective value of q (see text) The response was typical of that seen in 21 experiments Female cat 3 3 kg

This was always a *mean* pressure no pulsations were detectable From calculations in 19 expts no correlation was evident between the ratio DAP/FAP and the calculated total resistance of the vascular bed of the muscle or between the ratio and FAP During the course of an experiment this resting level of DAP commonly *changed but the direction and extent of change* with the exceptions noted later were apparently random and unrelated to obvious factors

Whatever might be the site in the vascular tree at which DAP was being measured (see Discussion) it was at a pre rather than postcapillary level as indicated by the pressure values This was confirmed by the observation that when blood was allowed to flow from the small artery cannula it was invariably bright red in three cases the oxygen saturation of the blood measured directly was 90 96 5 and 96 %

2 *Sympathetic vasoconstrictor response*

Stimulation of the sympathetic chain at the L4—L5 or L5—L6 level yielded a consistent reproducible pattern (Fig 2) in which DAP rose initially simultaneously with the decrease in femoral flow to its low point The pressure reached its peak in one half to one minute and immediately began to fall to a new lower level which might be as low as the original During the falling phase of DAP femoral flow tended to rise somewhat before attaining a relatively steady value On cessation of the sympathetic stimulation femoral flow rapidly rose to its original level usually manifesting a transient overshoot (reactive hyperemia) DAP decreased concomitantly and then returned to its control level along with the return of femoral flow I v administration of atropine 0 5 mg/kg *did not affect the response*

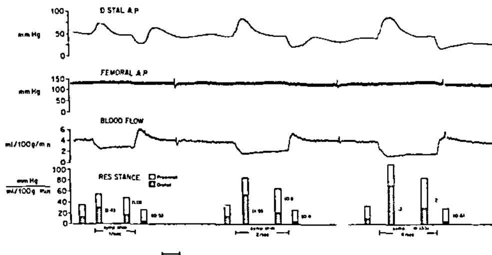


Fig 3 The effect of increase in stimulus frequency on the sympathetic vasoconstrictor response Note that both the total resistance and the value of q rise with the increase in stimulus frequency, the rise in q being particularly marked at the initial peak Designations as in Figure 2 Female cat, 3 3 kg

Calculation of proximal and distal resistances at several points throughout the response indicated relatively selective time dependent effects of the sympathetic innervation on the proximal and distal vessels (Fig 2) The initial increase in total resistance could be accounted for almost entirely by constriction in the distal segment, values of q ranged from 1 1 to 2 8 By the time the steady state reduction in flow was reached, however, resistance in the distal segment had returned towards or even below its control value (q 0 8—1 6) and the maintained elevation in total resistance could be ascribed almost entirely to constriction in the proximal segment Vasodilatation during the period of reactive hyperemia appeared to involve chiefly the more distal vessels (q = 0 4—0 7) In other words in the early phase of response to sympathetic stimulation, apparently the more distal vessels responded quickly with a substantial constriction After this transient period the distal vessels tended to relax and now the more delayed response of the proximal vessels became pronounced until it accounted for the major elevation in total resistance during the steady state With increasing intensities of sympathetic stimulation as tested in 3 expts total resistance increased as expected and the relative role of the distal vessels in the early phase became progressively greater (Fig 3)

In a few trials where constant flow perfusion was used the maximum change in total resistance with a given stimulus was essentially the same as in the constant pressure experiments In this situation of constant blood supply however the distal pressure tended to remain elevated for the duration of sympathetic stimulation rather than returning towards its initial value

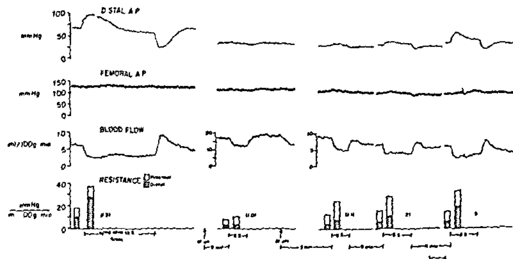


Fig 4 Effect of local exercise on the response to sympathetic vasoconstrictor fiber activation Only the values for resistances and q corresponding to the control (pre sympathetic stimulation) and to the peak DAP response are presented for each episode of sympathetic stimulation Between the first and second panels stimulation of the muscles was started as described in the text The second panel shows the response to sympathetic stimulation while the muscles had been exercising for 5 min note chanc ... panel two stimulation of the motor nerve ... 11 min (third fourth and fifth panels) ... of total resistance and q towards their in ...

3 *Effect of muscle exercise on the sympathetic vasoconstrictor response*

In these trials a standard stimulation of the sympathetic chain was repeated before during and several times following a 2 to 20 min period of exercise As indicated in the example in Fig 4 the total resistance (R_T) as was to be expected fell during exercise and was less affected by the sympathetic stimulation. To judge by the calculated values of q the response of the distal vessels was more markedly reduced than that of the proximal vessels during and for a considerable period after the exercise This trend was consistent in similar experiments in 7 animals and is shown graphical

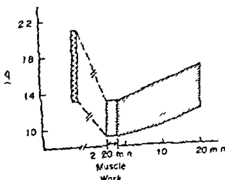
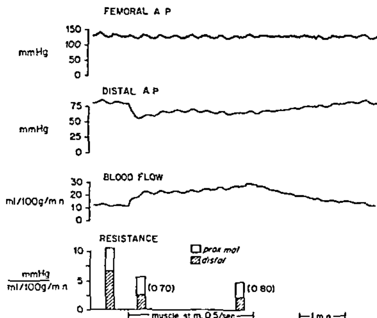


Fig 5 Changes in q accompanying sympathetic vasoconstriction during and after exercise summary of 7 experiments one of which is shown in Fig 4 The bar at the left represents the range of values of q during the initial peak response to sympathetic stimulation while the muscle was at rest The lowest portion of the graph is the range of q while the muscle was being stimulated over a period varying from 2 to 20 min The last rising portion of the graph is an envelope including values of q following cessation of motor nerve stimulation determined at varying intervals in the several experiments The fall in q during exercise indicates that the constrictor response of the distal vessels was more markedly reduced than that of the proximal vessels (see text)



ly in Fig 5 Even after a relatively short period of exercise q did not return to its control value for many minutes, often considerably later than the return of total resistance, as in the example of Fig 4

4 *Response to local exercise*

Within seconds of the onset of stimulation of the motor nerve, arterial flow increased and DAP fell reaching its low point in about one half minute (Fig 6) As stimulation continued DAP gradually rose somewhat while total resistance continued to fall Correspondingly the calculated resistance of the distal segment almost reached its minimum in the first one-half minute while that of the proximal segment decreased continually over about a 3 min period of stimulation

5 *Sympathetic vasodilator response*

Activation of the cholinergic vasodilator innervation in 3 cats accomplished by stimulating the sympathetic chain at L4—L5 or L5—L6 after intravenous administration of bretylium tosylate (10 mg/kg) led to a prompt hyperemia which typically died away during the stimulation (Fig 7) The dilatation involved the proximal vessels to a considerably greater extent than did a brief muscle contraction just sufficient to produce essentially the same extent of hyperemia, in the latter case, the dilatation was restricted almost entirely to the distal segment (Fig 7)

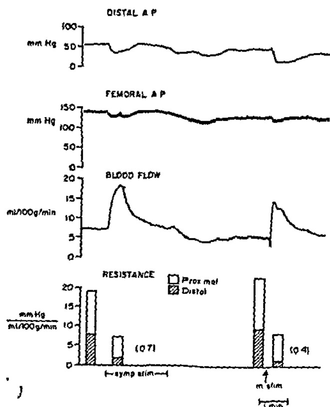


Fig 7 Comparison of effects of cholinergic vasodilator fiber activation and muscle exercise On the left, the sympathetic chain was stimulated after bretylium tosylate, 10 mg/kg, had been given i v On the right the motor nerve was stimulated briefly so as to induce a functional hyperemia of the same magnitude Note the greater fall in DAP in the latter case, and correspondingly lower value of q The dilator response to sympathetic stimulation was afterwards abolished by i v atropine, 0 5 mg/kg Comparable results were obtained in three other experiments Designations as in Fig 1 Female cat 2 5 kg

Discussion

The basic assumption underlying interpretation of the distal pressure measurement is that the cannulated small artery somewhere in its distal ramifications, connects through a collateral vessel with a parallel, and essentially equivalent, artery The small artery and its branches can then be considered as an extension of the cannula down to the point where the collateral meets the parallel arterial vessel, in which normal flow is proceeding We have been able to show directly (unpublished observation) that such collateral vessels do exist With the sural artery clamped proximally, yellow tinted vinyl acetate was injected into the cannulated small artery, after appropriate rinsing and blue vinyl acetate was injected into the femoral artery After the tissue was dissolved away in KOH, the distribution of the sural artery and the remainder of the femoral distribution were clearly evident Connections between the two differently colored arterial ramifications could be seen (The casts of these connecting vessels were about 80—100 μ in diameter which probably represents only an upper limit of the inside diameter of the collateral vessels, as the injection material was introduced under high pressure) This observation and the fact that arterial blood flowed retrogradely from the cannula (see Results, section 1) strongly substantiate the assumption that the meas[illegible] pr[illegible] ressure in the parallel arterial tree at the level of connection w[illegible]

It is not possible to localize anatomically with any certainty the level and type of arterial vessel whose pressure is being monitored in a particular experiment The levels of collateral connections probably vary among individual animals At most, one can stipulate that the pressure operationally defines a longitudinal partition of the vessels into a distal segment which includes the smallest arterioles and the precapillary sphincters, and a proximal segment which includes the larger arteries (The distal segment of course also includes venules and veins which in these experiments to judge by the lack of significant change in small vein pressure noted in Methods, did not contribute significantly to changes in distal pressure) In this connection, the observation of small artery pressures as low as 12—15 mm Hg in some cases is especially interesting Since this is still apparently a precapillary pressure, it indicates that at least in some animals, mean capillary hydrostatic pressure is lower than commonly supposed, but in the same range (10 0±2 0 mm Hg) as inferred by Prather (1968) from his observations on colloid osmotic and tissue hydrostatic pressures

The pressure gradient along the vascular bed defines the gradient of resistance Hence the partition according to pressure mentioned above is *pari passu* a partition of resistances into proximal (R_P) and distal (R_D) components whose sum is the total resistance (R_T) Direct inference from the simple vascular model indicates, then, that R_D/R_T represents the proportionate conturbution of the distal segment to the total resistance, and, hence, the way in which R_D/R_T changes reflects the relative participation of the distal vessels in the particular physiological condition An increase in R_D/R_T (*i e*, $q > 1$), for instance, means either that the distal vessels have constricted relatively more than the proximal vessels or that the proximal vessels have dilated relatively more than the distal Which of these is the case is determined by the change in total resistance that indicates overall constriction or dilatation

A possible alternative to the above interpretation is that the anatomical locus of the major patent collateral vessel is shifting upwards or downwards in a consistent pattern An increase on R_D/R_T (*i e* $q > 1$) as for example, during sympathetic vasoconstriction would then be interpreted as an actual opening of a previously closed collateral vessel which connects with a more proximal site where pressure is higher Such a dilatation of collaterals while other vessels are constricting would imply opposite neurogenic influences on the two sets of vessels Such a complicated organization of control over collateral vessels is most unlikely No evidence exists that these arterial or arteriolar branches have such a specialized innervation or that they differ otherwise from other vessels of comparable size

A final assumption inherent in this method is that changes in blood flow in the major supply artery (femoral) of the muscle reflect changes that are occurring fairly uniformly throughout the muscle One cannot measure the flow alone in that parallel vessel whose pressure is monitored through the postulated collateral Only by the assumption of a reasonable degree of uniformity can one calculate relative changes in resistance on the basis of relative change in flow in the vessel in question In a way analysis of this type exhibits many of the advantages and drawbacks of anal

of capillary pressure conditions in different physiological states by means of cannulations of single capillaries. Random variations of considerable magnitude no doubt occur, as a result, for instance, of uneven capillary flow but a uniform direction of results in a series of experiments can provide relevant information on the general quantitative and temporal characteristics of vascular responses proximally and distally to the point of measurement.

Changes in calculated resistances during sympathetic constrictor stimulation, to recapitulate, were an initial but transient increase primarily in the distal segment and a gradually increasing and sustained elevation in the proximal segment. During reactive hyperemia after stimulation was terminated, proximal resistance returned just to its control level, while distal resistance fell markedly below its control value and accounted for essentially all of the vasodilatation. The inferred slowly developing, ascending constriction that involves the larger arteries is quite the same as that directly observed by Gerova and Gero (1968) in large arteries (femoral artery of the dog) during sympathetic stimulation. They reported a much more rapid time course for the development of constriction in what they termed "resistance" vessels, i.e., those distal to the large arteries, than in the femoral artery. Moreover, as frequency of stimulation of the sympathetic was increased, the relative constrictor effect on the distal vessels as compared to the femoral was increased in a manner compatible to our observations (Fig. 3).

The initial constriction of the distal vessels, including terminal arterioles and precapillary sphincters, may result indirectly from diffusion of transmitter from nerve terminals situated on larger vessels, or by myogenic conduction distally from those vessels, to the extent that the muscle sheath is continuous, since a direct adrenergic innervation appears to be sparse in the smallest precapillary vessels (Fuxe and Sedvall 1965). Closure of the precapillary sphincters may also in part be a passive result of upstream constriction of the terminal arterioles. Following this initial constriction, a rapid adaption occurs, presumably through the action of locally produced dilator material, resulting in the opening of precapillary sphincters with a consequent tendency towards maintenance of diffusion capacity during the steady state of the sympathetic response. This is comparable with the report of Cobbold et al. (1963) that capillary filtration coefficient (CFC) of skeletal muscle at first decreases and then rises to or above its initial value during continued sympathetic stimulation. Return of capillary flow in skeletal muscle after 30 sec during prolonged sympathetic vasoconstrictor stimulations was directly observed by Eriksson and Lisander (1969) who also noted that the effect of constrictor fiber activation was evident on vessels of 15—300 μ diameter and predominant on those around 50 μ diameter.

While the general pattern of distal pressure response to constrictor stimulation is similar to that often seen by Abboud and Eckstein (1966) in the dog, in some of their experiments and those of Zimmerman (1966), in both of which constant flow perfusion was used, only a sustained increase in pressure occurred. This was likewise so in our trials with constant flow perfusion. In this situation, which is of

course unphysiological in that tissues are normally perfused at constant pressure when constrictor fiber activation does reduce flow, the postulated local vasodilator substances would accumulate to much less an extent, thus *the adaptation of* pre capillary sphincters *leading to their relaxation and the fall in distal pressure, might* not occur noticeably

The effect of muscle exercise on the response to sympathetic vasoconstrictor stimulation is an example of pronounced interaction between humoral and nervous factors *in control of vessels Our results coincide with those of Rein (1930) and* Kjellmer (1965) in demonstrating the marked reduction in intensity of the vaso-constrictor response during and for some time after exercise More than that in consonance with Kjellmer s (1965) observations our findings indicate that the antagonism occurs *especially* in the distal segment *i e*, the response to sympathetic activation of the distal vessels was more markedly reduced than that of the proximal vessels during exercise This might be expected in view of the primary action of the ' dilator metabolites at the site of their production presumably at the level of the smallest vessels which are in cloest proximity to the active muscle cells The lesser reduction of constriction in the larger more proximal vessels indicates that ascending dilatation is not as potent in counteracting the constrictor effect as is the direct action of dilator metabolites Nevertheless ascending dilatation in the larger vessels does take place to a considerable extent during muscle exercise in the presence of constrictor fiber activity in contrast in the duck the large arteries have a more dense and potent constrictor innervation which when activated produces intense constriction of these vessels that effectively prevents any ascending dilatation (Folkow *et al* 1966)

The development of ascending dilatation is illustrated in those experiments in which the muscle was stimulated continually for at least 2—3 min (Fig 6) Two discrete processes are shown by the DAP measurements an immediate dilata tion in the most distal vessels directly related to some local probably metabolic effect of the exercise and a gradually ensuing progressively ascending dilatation of the larger vessels most likely brought about by a myogenically conducted relax ation of the vascular smooth muscle (Hilton 1959)

When only a few second stimulus is given to the muscle resulting in a short lasting hyperemia equivalent to that produced by activating the sympathetic cholin ergic dilator innervation (Fig 7) essentially only the distal dilatation occurs Then by comparison the cholinergic dilatation is seen to involve chiefly the more proximal vessels *i e* not that segment that includes the precapillary sphincters This in ference is quite compatible with the observations of Hyman *et al* (1959) and Rosell and Uvnas (1962) that cholinergic d lator stimulation fails to increase trans capillary exchange of solutes and those of Djojosugito *et al* (1968) that CFC is decreased during cholinergic dilation

Thus the action of the cholinergic dilator innervation is seen to be more restricted than either that of the adrenergic constrictors or that of muscle exercise Both of the latter have an initial action on the distal vessels including the

sphincters, with a slowly developing ascending influence which ultimately involves the large arteries In the case of constrictor innervation, the effect on precapillary sphincters is evanescent, while the action of muscle exercise on the precapillary sphincters is continuous and relatively refractory to constrictor influence

These results and inferences are compatible with the concept that distribution of flow within the capillary bed *i e*, its diffusion capacity, is adjusted by local factors probably metabolic, towards an optimal level for the prevailing metabolic demand of the tissue When the flow distribution is restricted and total flow reduced by sympathetic vasoconstriction, one result is a rapid readjustment of capillary flow distribution through relaxation of precapillary sphincters with a consequent tendency towards restoration of adequate diffusion capacity On the other hand muscle exercise causes a primary relaxation of precapillary sphincters in response to the increased metabolic demand and this relaxation continues along with the demand The cholinergic dilator fiber system, having no direct effect on precap illary spincters neither increases diffusion capacity (Hyman *et al* 1959 Rosell and Uvnäs 1962) nor shows any effect on flow distribution or resistance when the vessels are already dilated during exercise (Hirvonen and Sonnenschein 1962)

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Inhibitory Control of the Abdominal Stretch Receptors of the Crayfish

III. The Accessory Reflex as a Recurrent Inhibitory Feed-back

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Abstract

JANSEN, J K S, A NJÅ, K ORMSTAD and L WALLOE *Inhibitory control of the abdominal stretch receptors of the crayfish III The accessory reflex as a recurrent inhibitory feed-back* Acta physiol scand 1971 *81* 472—483

e autogenetic activation of the thick accessory fibre of the stretch receptors has been intact receptors The steady state he sensitivity of the receptor over licted from the mechanism of the le effect of the feed back can be e receptor Some time-dependent d

The reflex activation of the thick accessory fibre from its own stretch receptor was discovered by Eckert (1961) The thick accessory fibre has an inhibitory effect on the stretch receptor (Kuffler and Eyzaquirre 1955) so the accessory reflex provides an example of a *recurrent inhibitory* feed back Such *recurrent* feed-back loops appear to be common in the coupling of the central nervous system The best known examples are perhaps the recurrent inhibition of cat motor neurones by means of motor axon collaterals (Renshaw *1941*) the recurrent inhibition of pyramidal cells of the cerebral cortex (Phillips 1959 Andersen Eccles and Loyning 1961) and the recurrent inhibition taking place in the thalamic relay nuclei (Andersen Eccles and Sears 1962) Numerous other groups of neurones are known to possess recurrent axon collaterals which possibly mediate recurrent inhibitory effects The functional significance of this type of neuronal coupling has not been established The abdominal stretch receptor of the crayfish offers the advantage that the recurrent inhibition occurs on the *input side of the system* *The receptor can be adequately* activated and the recurrent pathway consists of only two neurones and the signals of both can be recorded simultaneously

The present paper gives an account of the input output relationship of the autogenetic accessory reflex, and it is shown that it is possible to account quantitatively for the recurrent feed back effect from the central gain of the reflex and the inhibitory effect of the accessory fibre impulses There is sometimes in the present type of experiments also a reflex activation of the small accessory fibre (Jansen *et al* 1971 a) However, its effect is small and not included in this account Quantitative aspects of the reflex activation of the accessory fibres from neighbouring stretch receptors are dealt with in the subsequent paper (Jansen *et al* 1971 b)

Methods

The experimental setup and the methods employed are those described in the preceding paper (Jansen *et al* 1971 a)

Results

The slowly adapting receptor (MRO_1) gives a maintained discharge to a depolarizing transmembrane current step (Fig 1 C) The firing frequency increases with current intensity over the working range of the receptor This provides useful inputs for the reflex activation of the thick accessory neurone of the same stretch receptor (Fig 1 A, B) The accessory fibre activation is maintained for the entire duration of the MRO_1 discharge and it occurs after a latency of same 50 to 100 msec The unit reflexly activated in Fig 1 A and B (top traces) is functionally indentified by its association with large IPSPs in the MRO_1 These follow the action potentials in the nerve with a latency of some 5 msec and they are clearly seen in the membrane potential records (lower traces) except when they coincide with the MRO action potentials or the subsequent after hyperpolarization

The IPSPs interrupt the prepotentials leading up to the spikes of the MRO_1 and they hyperpolarize the receptor Thereby the following spike discharge is delayed and this represents the inhibitory effect of the accessory fibre The records of Fig 1

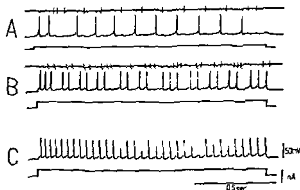


Fig 1 Reflex activation of the thick accessory fibre In each section Top trace action potentials of the dorsal nerve Middle trace membrane potential of the slowly adapting receptor Bottom trace the injected current Spikes retouched Receptor spikes in nerve record to faint to reproduce A B Intact reflex increasing intensity of depolarization current C After section of dorsal nerve Current intensity as in B Spikes retouched

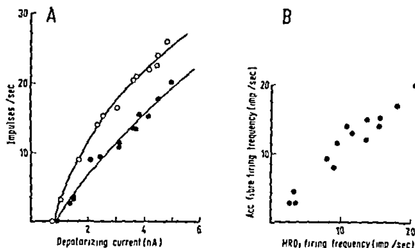


Fig 2 A Receptor firing fre[illegible]
rent ● Data obtained w
B Firing frequencies of t
of receptor Same prepar
pulses of 1 3 sec duration

illustrate the operation of a recurrent inhibitory feed-back with a single MRO_1 as input element and its accessory neurone as output element The effect of interrupting the feed back loop is seen by comparing Fig 1 B and C The MRO_1 activated by the same intensity of transmembrane current in both cases, before (Fig 1 B) and after (Fig 1 C) section of the dorsal nerve The average firing rate was appreciable reduced by the activity in the feed back loop

The effect of the feed back loop appears more clearly from the diagram of Fig 2 A This shows the firing frequency of the receptor during the last sec of the 1 3 sec depolarizing current pulses, plotted as a function of the current intensity The lower curve (●) gives the firing frequency of the receptor with intact feed back loop, while the upper curve (○) shows the results after section of the nerve The effect of the feed-back loop is to reduce the firing frequency of the receptor over the entire range examined As might be expected the frequency reduction is greater at higher levels of activity

The central gain of the autogenetic accessory reflex The records of Fig 1 also give the relationship between the increase in frequency of the thick accessory fibre and the corresponding increase in MRO_1 frequency This represents what might be called the central "gain" of the reflex and the plot of Fig 2 B shows that the firing frequency of the accessory fibre increased approximately linearly with the MRO_1 frequency of firing This was regularly found for accessory fibre frequencies up to some 30—40 imp/sec The same type of relationship has been found on activation of the accessory fibre from neighbouring MRO_1 (Jansen *et al* 1971 b)

In the example of Fig 2 B the gain of the reflex was very nearly one In other words each MRO_1 impulse elicited on the average one accessory fibre impulse This

was found also in several other preparations In other experim ts the was less, and in a few otherwise acceptable preparations the reflex was all lacking Some preparations with a good reflex initially might lo e th prolonged recording Since this is probably due to pathologic changes in ne for instance due to anoxia the average central gain of the acces ory ret minor interest The important observation is probably that the central gain often be as high as one and may remain stable during several hours of record

There was however another difference between preparations wich may e physiological significance In the example illustrated in Fig 2 B the accessory reflex was elicited as soon as the MRO_1 was activated In other preparations the reflex threshold was appreciably higher No reflex was elicited below some 10—15 imp sec [1] in the MRO_1 Above the threshold the gain of the reflex might be as high as one Such a system with a high threshold for the reflex might permit the receptor to operate without feed back inhibition at low levels of activity and with feed back on stronger excitation But we have no information about whether the observed differences in threshold are due to differences in the reflex coupling in the different preparations or whether they might be caused by some additional input not con trolled in our experiments which may bias the reflex loop

It should be pointed out that even though the reflex gain was frequently as high as one this does not imply any obvious time relation between the MRO_1 and ac cessory fibre impulses Usually the apparent central latency of the reflex was quite variable At certain levels of activity there might be a simple one to one driving around the reflex loop The record in Fig 1 A gives an exceptionally clear example of this In most other cases the firing was much more irregular and the time relation between individual MRO_1 and accessory impulses was quite complex (Fig 1 B)

Mechanism of the inhibition The inhibitory effect of the accessory IPSPs appears as a delay of the subsequent spike discharge of the MRO_1 Since the MRO_1s without a synaptic input are firing with a high degree of regularity to a constant stimulus the delay caused by an IPSP can be quite accurately assessed It has been shown to depend on the time of appearance of the IPSP within the MRO_1 interval The later the IPSP appears the greater is the delay and there is an approximately linear relation between the two (Perkel *et al* 1964 Jansen *et al* 1970 a) This type of behaviour finds its explanation in the intracelluar records of the IPSPs Each IPSP interrupts the prepotential of the MRO_1 and resets the membrane potential to a nearly constant value (Fig 3 B) This value is close to the equlibrium potential of the IPSP Consequently the IPSPs are small when evoked early after a MRO_1 discharge and they increase in amplitude with increasing depolarization during the prepotential After the resetting of the membrane potential the subsequent de polarization proceeds at a rate which is mainly determined by the rate of rise of the normal prepotentials (*i e* by the depolarizing current) This is illustrated in the plot of Fig 3 A which gives the delay from the peak of the IPSP to the next MRO_1 spike as a function of the rise time of the prepotential at that level of excitatory drive The dependency of IPSP amplitude on membrane potential

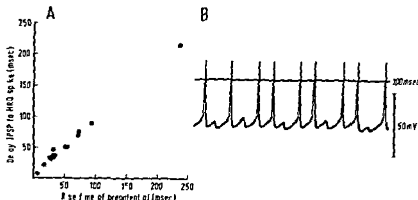


Fig 3 A Time from peak of IPSP to following spike (ordinate) ... of the prepotential at the same level ... record of receptor membrane pot... activated IPSPs determined by the ... at which they are evoked

the increasing duration of MRO_1 intervals with increasing delay of the inhibitory impulse (Jansen *et al* 1970 a), and the relationship illustrated in Fig 3 A suggests that this is valid at different levels of excitatory drives It is not suggested that this is a complete explanation But it will be shown in the discussion that it is sufficiently accurate to account for the behaviour of the MRO_1 with a fair degree of precision

The level of resetting by the IPSPs varies in different MRO_1s In some cells the resetting is beyond the afterhyperpolarization after the MRO_1 spikes by several mV In others the level of resetting has been less The smallest that we have seen in the present series of experiments is resetting to a level which was approximately 30 per cent of the difference between peak after hyperpolarization and the firing threshold of the cell With the present type of behaviour the degree of resetting should obviously determine the efficiency of the inhibition With a resetting to peak afterhyperpolarization, the longest MRO_1 intervals containing a single accessory spike should be, and have been found to be twice the intervals without inhibition An example of such behaviour has already been published (Jansen *et al* 1970 Fig 4) In MRO_1 with smaller degrees of resetting the inhibitory effects are smaller In the cell mentioned above with a resetting to 30 per cent above the peak after hyper polarization the maximal delay caused by an IPSP just before the expected MRO_1 discharge, was 70 per cent of the control interval We have at present no conclusive data on whether the difference in degree of resetting is due to differences in inhibitory equilibrium potentials in relation to the potential at peak after hyper polarization or whether it is due to quantitative differences in the conductance changes due to the inhibitory transmitter

The time dependency of the response The steady state properties of the accessory reflex which we have considered so far are conceptually simple, but do not reveal the full physiological significance of the reflex The time dependent properties of the response may well be equally important They can be elucidated by the study

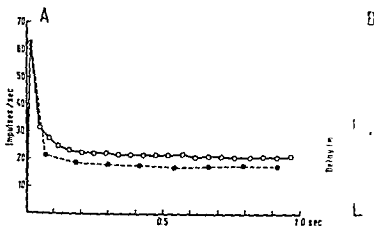


Fig 4 A Firing frequency of slowly adapting receptor during a n current starting at time zero ● Preparation with intact reflex arc ○ Reciprocal of interval for each spike is plotted for the denervated preparati for two and two intervals plotted for the intact preparation. B Delay times reflex Activation of receptor by depolarizing current steps Ordinates give ti e fr n first receptor spike to first large accessory spike of each step Corresponding current i ten m s along abscissa.

of the time course of the transient responses of the reflex A difficulty in this connection is the tendency for time locking between input and output spikes Such time locking can actually cause an increase in firing frequency of the MRO_1 with increasing inhibitory input, as pointed out by Perkel *et al* (1964) This problem frustrates a rigorous description of the time dependent response of the accessory reflex In spite of this we belive that a less formal description of the transient responses points out certain features that probably are meaningfull even though the full assessment of their quantitative importance has not been possible

The time dependent properties of the accessory reflex loop are appreciably influenced by three different factors The first is what might be called the central activation of the accessory neurone The second is the effect of the time delay of the reflex loop and the third is the inhibitory effect of the accessory fibre impulses on the MRO_1 The first of these is dealt with in the following publication (Jansen *et al* 1971 b)

The loop delay The effect of the loop delay is immediately obvious from the records of Fig 1 A and B To a step input there is a delay of some 50—100 msec from the first MRO_1 spike to the first accessory spike. Consequently, the initial part of the response is not influenced by the recurrent inhibitory feed back This is perhaps better illustrated in the plot of Fig 4 A, which gives the frequencies of firing of a MRO_1 to a similar step input. There is clearly a relatively greater initial overshoot in the response with intact feed back loop Therefore the response of the feed back loop will depend upon the frequency of the input signal The frequencies of the input which wil

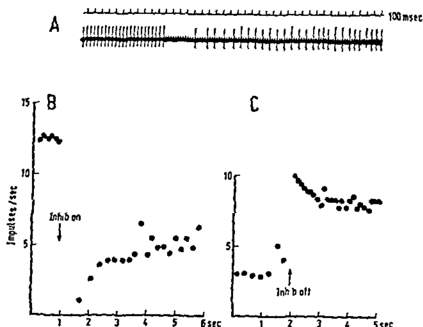


Fig 5 Transient responses to step changes in inhibitory input Data from slowly adapting receptor of VIIIth thoracic segment A Sample record of receptor activity (large spike) oked by stretch Constant frequency activation of thick acessory fibre (small spike) caused harp decrease in firing frequency B Inhibitory on transient Instantaneous frequency plotted nst time C Inhibitory off transient

the duration of the loop delay This is not constant for the accessory reflex Fig 4 B gives the observed delays at different levels of activation Two points should be mentioned There is a high degree of scatter in the results at any level of excitation and secondly there is a tendency for the delay times to be longer at low levels of activity Both these factors may be important

The time dependency of the inhibitory effect The time dependency of the inhibitory response is most clearly seen in the effect of a step inhibitory input on the firing frequency of a stretch activated MRO_1 The thoracic streth receptors are favourable preparations for this type of experiment The piece of nerve connecting the MRO_1 and MRO in the VIIIth thoracic segment is sufficiently long for antidromic activation of the accessory fibre (Kuffler and Eyzaguirre 1955) and its inhibitory effect on the MRO_1 discharge can be determined An example is presented in Fig 5 A The MRO_1 was stretch activated and firing at a frequency of about 20 imp/sec Antidromic activation of the thick accessory fibre at a rate of 30 sec^{-1} silenced the discharge immediately and this was followed by a fairly slow partial recovery of the firing Such a transient overshoot of the inhibitory effect was seen in all preparations of this type and the time course of recovery is illustrated in Fig 5 B It appears that most of the recovery was completed in approximately three seconds

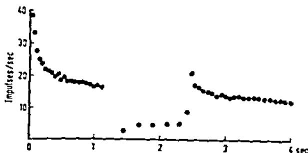


Fig 6 Transient responses of slowly adapting receptor to current steps Instantaneous frequency plotted against time Receptor depolarized by 5 nA current at time O From 1 2 to 2 5 sec there was a step reduction in current to 2 nA

On account of the difficulties with time locking mentioned above, we have not attempted to measure the time course of recovery systematically The duration of the initial silence increased with increasing frequency of the inhibitory input at a given background rate of firing The subsequent recovery phase had a fairly constant time course for each cell, but might differ appreciably between cells Most of the recovery might be completed already after one second

At the end of an inhibitory step input there was usually a rebound increase in firing frequency of the cell as reported previously by Kuffler and Eyzaguirre (1955) This rebound usually had a time course comparable to that of the initial overshoot, as illustrated in Fig 5 C the data of which are from the same cell as Fig 5 B The amplitude of the rebound effect increased with the degree of inhibitory suppression of MRO_1 firing

Several factors may contribute to the inhibitory transient responses The similarity in time course of the initial transient and the rebound suggests that the spike generating mechanism of the MRO_1 may contribute substantially This has been tested by examining the time course of the response of the MRO_1 to positive and negative steps of depolarizing currents An example of the response is shown in Fig 6 At the onset of the current step the cell started firing at a frequency of about 40 imp/sec The frequency fell continuously during the first second to half the initial value A step reduction in depolarizing current was applied at this time This may be thought of as analogous to an inhibitory input It appears that the frequency of firing was reduced with an initial overshoot and a subsequent recovery of firing At the return of the initial current intensity there was a rebound increase in firing frequency which subsided with a time course similar to that of the initial on-transient

The present material is too limited to argue that the initial overshoot and the rebound of the inhibition are identical to the corresponding phenomenon of the spike generating mechanisms illustrated in Fig 6 But the general similarity in time course and amplitude certainly indicates that the properties of the spike generating mechanism are probably a main factor determining the time course of the inhibitory response

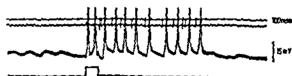


Fig 7 The accessory reflex as a positive feedback Slowly adapting receptor penetrated with chloride filled electrode Short current step (bottom trace) The receptor activated the thick accessory fibre (top trace) which gave depolarizing IPSPs firing the receptor The spontaneous depolarizations preceding receptor activation are reversed spontaneously occurring IPSPs

Reversal of feed back effect The polarity of IPSPs of the MRO_1 can be reversed by chloride ions injected intracellularly into the receptor (Hagiwara Kusano and Saito 1960 Janset *et al* 1971 a) The depolarizations produced by the large accessory IPSPs can then be sufficient to discharge the MRO_1 This will change the sign of the feed back signal in the reflex loop from negative to positive and one expects that the loop would be unstable and oscillate An example of such behaviour is illustrated in Fig 7 The MRO_1 was discharged twice by a short depolarizing current pulse This input activated the thick accessory fibre (top trace) which generated a large depolarizing IPSP and fired the receptor This gave a second accessory fibre output and the impulse activity continued in the self re exciting loop at a rate termined by the loop delay The oscillations stopped when the last MRO_1 impulse did not elicit an accessory fibre discharge Such behaviour was often seen when potassium chloride filled electrodes were used for recording

Discussion

The mean steady state effect of the accessory reflex is to reduce the gain of the MRO_1 (Fig 2 A) It appeared that the observations on the mechanism of the inhibition (Fig 3) and on the central gain of the reflex (Fig 2 B) enables one predict quantitatively the effect of the feed back loop with a fair degree of accuracy

Let the level of resetting caused by an IPSP be equal to the peak after hyper polarization of the MRO_1 spikes Then the shortest possible delay caused by an IPSP will be near to zero and the longest approximately equal to a MRO_1 control interval If we assume that the average time of occurrence of an IPSP is in the middle of a MRO_1 control interval the delay caused by an IPSP will on the average amount to 50 per cent of the MRO_1 control interval

Fig 8 shows the closed loop response of a MRO_1 in which the level of resetting caused by an IPSP was close to peak after hyperpolarization following each MRO_1 spike The gain of the reflex was approximatley 0 5 that is two MRO_1 spikes gave one accessory spike The open loop response obtained after the MRO_1 had been denervated is also plotted For this receptor the calculated duration of an interval containing an IPSP will be 150 per cent of the MRO_1 control interval (*c*) With the

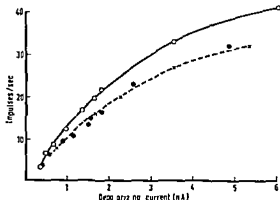


Fig 8 Comparison of observed (●) and computed (×) closed loop response of slowly adapting receptor (O) Response of receptor after nerve section The central gain of this receptor was 0 5 and the IPSPs caused a resetting to peak after hyperpolarization

observed central gain of 0 5 the calculated average duration of intervals with the reflex intact becomes

$$\frac{t + 1\,5\,t}{2} = \frac{5}{4}t$$

The calculated closed loop response is given in Fig 8 (×) and there is an acceptable agreement with the experimentally observed closed loop response (●) The closed loop response of other receptors with different central gain factors or resetting could also be predicted with about the same degree of accuracy The accessory reflex thus provides an example of a simple recurrent feed back reflex for which it appears possible to account for the loop effect in terms of the reflex coupling and the mechanism of inhibitory action

It is of some interest to determine the effect of this model of inhibition on different experimental situations which have been used to describe inhibitory effects quantitatively Consider for instance a constant frequency of firing in the accessory fibre Acting at different levels of receptor activity such a constant inhibitory input will cause an approximately constant reduction in receptor firing frequencies over its entire working range An increasing inhibitory input will furthermore cause an approximately linear reduction in the firing frequency of the receptor and these predictions have been experimentally confirmed (unpublished observations) These results are useful for the assessment of the overall effect of the interaction of several accessory reflexes in neighbouring abdominal segments (Jansen *et al* 1971 b) Granit and Renkin (1961) found a comparable linear behaviour of the recurrent inhibition of cat motoneurones but in this case the inhibitory mechanism is probably entirely different It is of interest that the present stretch receptors obtained a similar inhibitory feed back with a single inhibitory nerve fibre

With regard to the transient responses of the accessory reflex loop, the main merit of the present observations is to point out the various factors that contribute to the response of the receptor under dynamic conditions These various time dependent factors are commonly seen in the response of neurones and will normally have to be taken into account in any consideration of dynamic behaviour of chains of neurones The steady state effect of the recurrent inhibitory feed back is to reduce the gain of the receptor With the delay in signal transmission around the loop the early parts of the response will not be affected by the inhibition (Fig 4 A) The loop will act as a band pass filter The input frequency which is most effectively transmitted is determined by the loop delay Consider a cyclic movement If the receptor is maximally released at the moment when the recurrent feed back has its maximal effect, the inhibition will increase the amplitude of the output at this frequency This will occur when the period of the movement is twice the loop delay For the accessory reflex (Fig 4 B) this should take place with movements of a frequency of 5 to 10 per sec The response of the complete reflex loop will be determined also by the properties of the central coupling of the reflex, and this will be dealt with in the following paper (Jansen *et al* 1971 b)

The initial overshoot and subsequent rebound of the inhibition will also contribute to the frequency response of the intact reflex These inhibitory transient responses appeared to be largely due to the properties of the spike generating mechanism of e receptor (Fig 6) From this kind of transient responses of the spike generating mechanism, one would expect a frequency response with gain increasing with increasing frequency This is in conflict with Terzuolo *et al* (1968) who reported a flat frequency response for the encoder of the stretch receptor over the relevant range of frequencies One might suspect that the present transient responses were due to injury by the microelectrode but this appears unlikely since Nakajima and Onodera (1969) found quite similar transient responses of the spike generating mechanism also when the receptor was stimulated with extracellular electrodes

A possible factor is the electrogenic Na pump causing the long lasting post tetanic after hyper polarizations of these receptors (Nakajima and Takahashi 1966) This will be activated by the sodium influx during the early spikes and will act as a main tained hyper polarizing current as long as the cell is activated It is doubtful how ever whether the effect of the pump would be sufficiently rapid to explain the present transients (Fig 4 and we have tried to block the effect of the pump by the addition of ouabain In the period before all spike activity is blocked this causes a reduction in the firing threshold of the cell without marked effects on the time course of the transient responses

To summarize the main points of the present discussion The steady state effect of the accessory reflex is to reduce the sensitivity of the receptor over its entire working range The loop delay time the central coupling of the reflex and the inhibitory transient responses will introduce band pass filtering into the response of the system This filtering effect will accentuate the signal transmission in a frequency range that may be physiologically important

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Inhibitory Control of the Abdominal Stretch Receptors of the Crayfish

IV. Quantitative Description of the Central Coupling of the Accessory Reflex

By

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Abstract

Jansen, J K S, A Njå, K Ormstad and L Walløe *Inhibitory control of the abdominal stretch receptors of the crayfish IV Quantitative description of the central coupling of the accessory reflex* Acta physiol scand 1971 *81* 484—491

The two accessory neurones have been reflexly activated from neighbouring ipsilateral stretch receptors The firing frequencies of both increase with increasing input frequencies up to a *saturation level which was higher for the thick accessory neurone Inputs from different seg*ments are summated The frequency response of the reflex activation of the large accessory neurone has a maximal gain between 2 and 5 Hz This is within the frequency range of normal movements

A quantitative description of the reflex output of the two accessory neurones to different inputs are necessary to assess the significance of these reflexes

This paper gives an account of the reflex activation of the accessory fibres from stretch receptor afferents of the neighbouring abdominal segments

Methods

The experiments were performed on the isolated abdomen of fresh water crayfish (*Astacus fluviatilis*) The experimental conditions are presented in a preceeding paper (Jansen *et al* 1971 a) The dorsal nerves were cut peripherally and the central end dissected free to the lateral border of the extensor muscle Each of the nerves were lifted into a layer of paraffin on a pair of platinum electrodes The electrodes were used for electrical stimulation of the nerves and for recording of their activity In the present experiments repetitive electrical stimulation was performed at intensities just above threshold for the afferent axons from both the slowly and the fast adapting stretch receptors The identity of the reflexly activated spikes was [illegible] distribution and time course of the response [illegible] ctrical stimuli which were employed to the [illegible] ceptor afferents (Jansen *et al* 1970) The input pulse train and the [illegible] spikes were recorded on magnetic tape and on

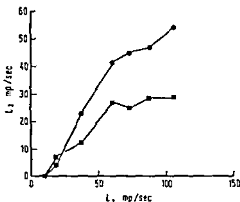


Fig 1 Output from the thick and thin accessory neurone as functions of a constant reflex input Abscissa Frequency of electrical stimulation of the dorsal nerve on the left side in the second abdominal segment Both the stretch receptor afferents were stimulatew Ordinate Frequency of firing of the two reflexly activated spikes in the dorsal nerve on the left side in the third abdominal segment Circles Thick accessory neurone squares thin accessory neurone Frequencies measured over 200 msec 0 5 sec after the initial on transient

moving film The magnetic tape was later played back through an electric counter which counted the number of unitary time intervals (usually of 1 6 m sec duration) that occurred between the action potentials These series of numbers were transfered to a Nord I computer via perforated paper tapes

Results

The thick accessory neurone is reflexly activated from the stretch receptors in the neighbouring segments in the crayfish abdomen During maintained activation the frequency of firing of the thick accessory neurone usually increased linearly with the input frequency in the range from 10 to 50 imp/sec In many preparations no reflex was activated with input frequencies below 10 imp/sec A typical example is shown in Fig 1 In this experiment the relationship between the increase in frequency of the accessory neurone and the corresponding increase in input frequency called the steady state gain of the reflex was about 0 8 In most other experiments it was less The gain always decreased with increasing input frequencies above some 50 imp/sec The maximum output was usually about 50 imp/sec and it was obtained with an input frequency of about 100 imp/sec For higher input frequencies the reflex was saturated and there was no increase in output frequency The gain of the reflex varied considerably from one experiment to another and between different input nerves in the same preparation In general the reflex couplings from the ipsilateral stretch receptors in the two neighbouring segments were about equally strong Reflexes from more distant ipsilateral stretch receptors and from contralateral stretch receptors usually had lower gain

The thin accessory neurone may also be reflexly activated from ipsilateral stretch receptors in much the same way as the thick accessory neurone (Jansen *et al* 1971 a Several preparations however showed no reflex activation of thin accessory neurones although the thick accessory neurones could be strongly activated One systematic difference between the two reflexes seems to be that the gain of the reflex to the thin accessory neurone is less and that it is saturated already at output frequencies of about 25 imp/sec A typical example is shown in Fig 1

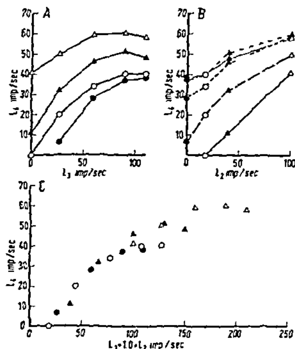


Fig 2 Output from the thick accessory neurone reflexly activated from two input nerves

A Abscissa Stimulation as in Fig 1 in the third segment Ordinate Frequency of firing of the thick accessory neurone in the fourth segment From top Simultaneous stimulation in the second segment with frequencies 100 imp/sec 40 imp/sec 18 imp/sec and 0 imp/sec respectively

B The same experimental data as in A plotted as a function of the frequency of stimulation in the second [illegible] and 0 imp/sec

C Again the same experimental data as in A and B but this time plotted as a function of a weighted sum of the two stimulation frequencies as explained in the text

Summation of input The output frequency of the thick accessory neurone was also investigated with simultaneous stimulation of two input nerves Fig 2 shows results from one typical experiment In this preparation the thick accessory neurone in the fourth segment was activated ipsilaterally both from the third and second segments A and B in this figure show the results from experiments in which the dorsal nerves in the second and third segments were stimulated simultaneously with different frequencies The results are typical for all experiments of this kind If both stimulus frequencies were relatively high (50 imp/sec or higher) the output frequency was much less than the sum of the output frequencies elicited by each input nerve when it was stimulated alone If one or both input frequencies were lower than threshold for the elementary reflexes the output frequency when both input nerves were stimulated was considerably higher than the sum Let the following equation define a new summed 'input frequency'

$$f_s = f_1 + af_2$$

f_1 and f_2 are the input frequency in each of the two dorsal nerves In all experiments it was possible to determine one number a between 0 0 and 1 0 which transformed the results of that experiment (as shown in Fig 2 A and B) to a simple relationship between the new input frequency (f_s) and the output frequency One example is shown in Fig 2 C In this particular preparation $a = 1\ 0$ a corresponds to the ratio between the gains of the two elementary reflexes Below the level of saturation the relationship obtained with input defined as this weighted sum of the individual in

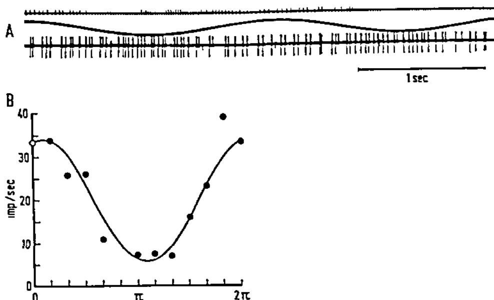


Fig 3 *A* The primary data for the frequency analysis of the reflex as recorded on moving film and on magnetic tape Traces from the top 1 Stimulus pulses with sine-wave modulated frequency as applied to the dorsal nerve on the left side of the fourth segment. 2 Modulating sine wave 3 Output spikes in the thick accessory neurone in the dorsal nerve on the left side in the third segment An occasional spontaneous spike can also be seen in this tracing

B Output from a reflex from the fourth to the third segment (right side) averaged over 48 cycles in the manner described in the text Input modulation frequency 4 cycles/sec total observation time 11897 msec total number of output spikes 199

Circles Mean frequencies in each of the twelve bins

Line the best fitted sine-wave Parameters of this sine wave mean frequency 20 1 imp/sec amplitude 14 1 imp/sec phase −16 7°

puts is equal to the input-output relationship obtained with input from one nerve alone The value of *a* was often close to one when ipsilateral nerves just in front and just behind the recorded segment were stimulated For the interaction between a neighbouring and a more distant nerve the value of *a* was usually less than one Values of about 0 3 were common This is in agreement with less extensive data presented earlier (Jansen *et al* 1970 Fig 6 A)

The saturation of the response is a striking feature of Fig 2 A But apparently the *saturation level is not constant With an additional input it was possible to increase* the frequency of firing still further This may possibly suggest that the two inputs act at different receptive regions of the accessory neurone

Sinusoidal inputs There are important features of the accessory reflex which are not revealed by investigations of the static response The most efficient way to study the dynamic properties of the reflex is to use frequency analysis and determine the transfer function of the reflex This method has however one serious limitation The system has to be linear or at least approximately linear for small t a

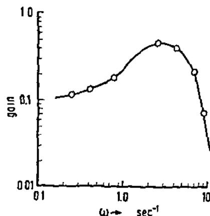


Fig 4 Plot of the frequency response of the accessory reflex Ordinate gain defined as the ratio output amplitude (imp/sec)/input amplitude (imp/sec) Abscissa modulation frequency Same experiment as in Fig 3 B Mean output frequency of firing in this experiment was about 20 imp/sec

tudes One particular type of non linearity has been found in the central reflex which is particularly troublesome for frequency analysis The output spikes in the thick accessory neurone have a tendency to time-lock with the input spikes, either in a one to one fashion or in a one to two fashion The time-locking is only present in me preparations and in these preparations only for some input frequencies In spite of these difficulties, we have tried to use the methods of frequency analysis to study the dynamic properties of the central reflex Fig 3 A shows an example of the primary data obtained from these experiments The dorsal nerve in one segment is stimulated repetitively The frequency of the pulse-train is modulated by a steady state sine-wave The mean pulse frequency, the modulation amplitude and the modulation frequency could be varied independently The output frequency of the ipsilateral dorsal nerve in the neighbouring segment was averaged over a number of cycles in the following way The modulation period was divided in a number (usually 12 or 40) of equally long bins In each cycle the time from the beginning of that cycle to each spike occurring in it, was calculated by the computer For each spike the inverse of the immediately preceding interspike interval was also calculated This instantaneous frequency was assigned to the corresponding bin When the computer had read through all the cycles which were to be used the average frequency for each bin was calculated The corresponding curve was fitted with a sine curve with the same frequency as the input by a leastsquares program The mean pulse frequency the amplitude and the phase shift in the output was thus estimated by the computer A typical example is shown in Fig 3 B

For small input amplitudes and low modulation frequencies most of the reflexes seemed to be linear 1 The output frequency curves had a sine wave shape 2 The amplitude of the output increased proportionally to the amplitude of the input In this small amplitude range the output amplitude is a characteristic function of the modulation frequency One example is shown in Fig 4 The reflex seems to operate like a band pass filter turned to input frequencies in the range from 2 to 5 cycles/sec

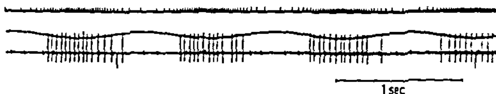


Fig 5 A non linear reflex Tracings as in Fig 3 A

There are, however, at least two different kinds of non linearities in this system One is caused by the time locking mechanism When one to one driving occurs the output from the reflex will of course be very similar to the input with gain equal to one The trouble is that for a given reflex time locking occurs only for some input frequencies and amplitudes thus causing non linearities in the system In some preparations time locking distorted the output and gave inconsistent responses which have been discarded

The reflex system also gradually becomes non linear when either the input amplitude or the modulation frequency is increased This kind of non linearity is illustrated in Fig 5 The figure shows the output from a non linear reflex to a sine-wave modulated input The output frequency curve usually had a triangular form with a sharp rising phase and then first a slow and then a rapid fall in frequency In extreme cases the output curve was rectangular with the accessory neurone firing with a constant and high frequency during one part of the input cycle and being silent during the rest of the cycle When a reflex became non linear by increasing the input amplitude the output frequency was always greater than expected for the linear model during the rising phase of the input sine wave and smaller during the falling phase

Discussion

The experiments have shown that there is a simple relationship between the steady state output from the thick accessory neurone and the frequency of the input in the neighbouring dorsal nerves Above a certain threshold the output frequency increases linearly with the input frequency with gain almost as high as one The curve flattens off for higher input frequencies The same type of relationship is obtained when two input nerves are stimulated simultaneously if a new input frequency is calculated as a weighted sum of the two individual inputs It is tempting to suggest that a similar transformation of the inputs could be done if more than two input nerves were stimulated but these experiments have not yet been done In the present experiments the afferent fibres of both the slowly and the rapidly adapting receptor (called MRO_1 and MRO_2 respectively), are stimulated simultaneously in the dorsal nerves The thick accessory neurone is reflexly activated from both these afferents and in a previous paper we have shown that a summation of reflex activity similar

to the one described in this paper, takes place between MRO_1 and MRO_2 (Jansen *et al* 1970, Fig 2 and 4)

The accessory neurone is accordingly activated by a fairly large number of input signals from a series of stretch receptors extending in pairs along the length of the abdomen In many physiological movements most of these would presumably be activated The most powerful excitation of a given accessory neurone would be from its own MRO_1 The gain of this autogenetic reflex is often as high as one (Jansen *et al* 1971 b) The signals from the MRO_2 is usually much less efficient (Eckert 1961, Jansen *et al* 1970) The reflex connection from a neighbouring MRO_1 is usually less powerful than the autogenetic connection It should be recalled that gains as high as 0 8 in neighbouring reflexes (Fig 1) was obtained by coactivation of the two MRO afferents More distant stretch receptors are even less efficient, and the same applies to contralateral receptors (Jansen *et al* 1970) With the aquired knowledge of the summation of inputs, described above, and from the inhibitory action on the receptors (Jansen *et al* 1971 b) we can consider the overall effects of the accessory reflexes of all the segments During abdominal flection the accessory reflexes of several neighbouring segments will be activated On account of the higher gain of the autogenetic accessory reflex the absolute reduction in MRO_1 firing frequency will be greatest in the segment most strongly activated But the relative reduction in firing frequencies will be greater in neighbouring segments on account of reciprocal inhibitory reflexes and hence the overall effect will be an accentuation of the relative differences in the degree of activation of the various segments This increased contrast in the afferent signal may possibly be of importance in the motor control of the animal, just as the comparable lateral inhibition in the eye may contribute to visual resolution (Hartline and Ratliff 1957)

Within the linear range a simple model of the synaptic coupling could explain the interaction between the stretch receptor inputs to the accessory neurone The essential features of such a model would be 1 Direct connections to the thick accessory neurone from both the MRO_1s and the MRO_2s in the same and neighbouring segments 2 EPSPs with different amplitudes produced in the postsynaptic cell from spikes in the different input neurones 3 Post synaptic summation of the unitary EPSPs to some threshold and resetting of the membrane potential (Walloe *et al* 1969) A more complicated model would be required to explain the different levels of saturation illustrated in Fig 2 A

The non linearities observed in the central reflex rise two groups of questions 1 What kind of mechanisms in the synapses produce the non linearities? 2 Do the non linearities have functional significance?

Part of the non-linearity observed with large input amplitudes may be explained as saturation of the reflex In addition it is necessary to postulate some mechanism which is sensitive to the rate of change of the input This component will have a phase advancing effect Such phase advancing mechanisms may be of importance to the animal, since the total delay in the reflex loop due to conduction and synaptic delays, is rather large As discussed in the preceding publication (Jansen *et al*

1971 b) the loop delay will also introduce band pass filtering into the response of the stretch receptor accentuating the responses in a frequency range between 5 and 10 per sec The frequency response of the central reflex will increase the response amplitude of the stretch receptor in this range still further It is perhaps significant that this corresponds to the frequency of the tail flip of the crayfish (Roberts 1968) The functional implications of the time locking mechanism i more difficult to evaluate It is probably less pronounced in a physiological situation with less synchronous inputs

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Electrophysiological and Morphological Development of Leg and Neck Muscles in the Rat

By

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Abstract

BOETHIUS, J *Electrophysiological and morphological development of leg and neck muscles in the rat* Acta physiol scand 1971 *81* 492—507

Membrane potentials were measured in the neck, sartorius and gastrocnemius muscles of post natal rats The potential rose from a constant value of 60—70 mV up to adult values of 80—90 mV This increase taking about 5 days was earliest in the neck and latest in the gastrocnemius muscle In the electromyograms of newborn rats the voltage of the motor unit potentials was higher and the duration shorter in the neck than in the gastrocnemius muscle Monophasic motor unit potentials were more common in the gastrocnemius than in the neck muscle Later the gastrocnemius potentials became comparable to those of the neck musculature In the neck musculature myotubes (3—27 %) were only found during the first day In the sartorius and gastrocnemius muscles about 20 % of the muscle cells were myotubes at birth In each muscle the myotube myocyte transformation was completed on the day when the rise in membrane potential started The correlation between the development of the membrane potential and the myotube-myocyte transformation was also investigated in muscles denervated at birth and subsequently reinnervated In such muscles the percentage of myotubes was 20—27 % at a time when the membrane potentials were of adult magnitude

In the early part of its ontogeny the muscle cell develops from the premyoblast to the myotubular stage (Tello 1922, see also Boyd 1960) The subsequent phase of development is characterized by the transformation of myotubes into myocytes In the chick embryo, this phase is also characterized by an increasing functional and morphological interaction between the motor nerve and the muscle cell The electromyogram attains an essentially adult pattern (Boethius 1967) and an accumulation of acetylcholinesterase can be demonstrated at the neuromuscular junction (Mumenthaler and Engel 1961 Filogamo and Gabella 1967)

In embryonic chicks the muscle membrane potential is unchanged and rather low during early developmental stages (Boethius and Knutsson 1970) At about the time when the myotubes are transformed into myocytes there is a sharp increase in membrane potential Thus there is a temporal correlation between the development of the membrane potential, the electromyogram the motor end plate and the myotube myocyte transformation

The present study is an extension of the previous studies on the chick emb . It was undertaken mainly in order to determine whether the above-mentioned tempo correlations could also be found in mammals, and whether the rise in mem potential and myotube-myocyte transformation were functionally coupled or occur independently of each other

Postnatal rats were chosen as experimental animals, the morphological deve ment of the muscle cells and end-plates in this species having been thoroughly de scribed (see e g Csillik 1960, Teravainen 1968 a, b, Kelly and Zacks 1969 a, b and because an increase in muscle membrane potential reportedly takes place after birth (Fudel-Osipova and Martynenko 1962).

Methods

Membrane potentials The experiments were performed on 14 Sprague-Dawly rats during their first 12 postnatal days The animals anesthetized with urethane (20 %, 7 5 ml/kg body weight) and pinned to a cork board During experimentation measurements were first made on the gastrocnemius muscle The skin overlying the muscle was removed together with the . The exposed muscle sur rane potentials were first ctrode slowly through the neck muscles were then embrane potentials in the sartorius muscle were measured in the same way In each muscle about 10 electrode tracks were made during an experiment, yielding a total of 100—200 measured potentials

During the experiment, the rectal temperature was kept as low as 34—35° C, the animals surviving the experimental procedures better at this temperature The low temperature might

reproducible

those actory penetration properties

The microelectrodes were connected to a DC-coupled preamplifier with an upper limiting frequency of 10,000 Hz The signal was then fed into an oscilloscope (Tektronix 502) and registered on film

removing the fascia. Accordingly these low potentials were also excluded

Stable membrane potentials Stable membrane potentials were obtained with the aid of a micromanipulator driven at high acceleration in 2 μ steps by a step-motor

Mean maximum membrane potentials In an attempt to exclude the low potentials caused by faulty cell penetrations, the membrane potential was alternatively measured as the highest potential in each electrode track, here called the maximum membrane potential For each muscle the mean maximum membrane potential ($n = 8—17$) was then calculated

Electromyogram Electromyograms (EMGs) were obtained from the gastrocnemius and neck muscles of 10 unanesthetized rats (age 0 to 10 days) The measurements were made a

concentric electrode used in clinical routine work (Disa, 9013 K 0511) This comparatively large electrode was used in order to facilitate comparison with a previous work on the EMG of chick embryos (Boethius 1967) The electrode was connected to an AC-coupled preamplifier (band width 5—10,000 Hz) The EMGs were displayed on the oscilloscope and recorded on film The duration of the motor unit potentials was measured according to the criteria of

two distances, one representing the longest straight line between two points on the circumference of the cell and the other the largest "diameter" perpendicular to this line The muscle fibres tended to occur in groups of either comparatively large or comparatively small fibres A fairly unbiased sample could, however be obtained by defocussing the preparation and moving the microscope stage at random, then focussing again and measuring on the fibre in the centre of the visual field

scribed above

Denervations The experiments were performed on 4 rats The animals were anesthetized by cooling to 6—10° C An incision was then made dorsally in the right thigh The denervation was performed by removing about 3 mm of the sciatic nerve At a later date, membrane potential and percentage of myotubes were determined for the denervated gastrocnemius in the ianner described above Measurements obtained on the contralateral gastrocnemius muscle ere used as controls

Results

Membrane potentials Fig 1 illustrates the distribution of the membrane potential at different ages For each age, all potentials were recorded in the same animal with one exception the neck potentials, marked with an asterisk in the ten day row, are from a 9-day old rat At all developmental stages the potential values were roughly normally distributed The standard deviations were 4—9 mV, *i e*, values of the same order of magnitude as those of adult rats (Li, Shy and Wells 1957, Muscholl 1957, Zierler 1959).

Fig 1 also shows that during the first two postnatal days the membrane potentials in the neck muscle increase In the sartorius muscle, a similar shift can be seen between the 4th and the 8th day after birth The gastrocnemius potentials, finally, are seen to increase some time between the 4th and the 10th extra-uterine day

Mean membrane potentials Fig 2 illustrates the development of the mean membrane potentials A general increase in mean value took place between the 1st and the 10th day in all three muscles This increase was earliest in the neck muscle (Fig 2 A), the potential of which rose from a value of about 70 mV at birth and reached the adult value of about 80 mV on the 2nd or 3rd day of life The sartorius value (Fig 2 B) was about 60 mV at the time of birth During the subsequent development it rose up to the adult value of 80 mV, which was reached on the 8th day At the time of birth, finally, the mean membrane potential of the gastrocnemius muscle (Fig 2 C) was roughly the same as that of the sartorius muscle, *i e*, about 60 mV Between

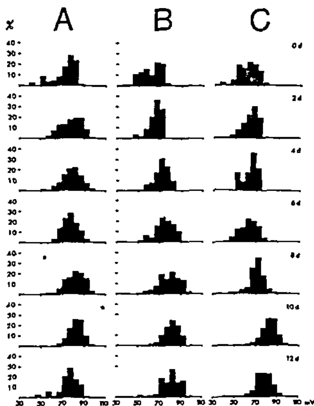


Fig 1 Membrane potential distributions at various ages A: neck, B sartorius and C gastrocnemius membrane potentials At every age the potentials were obtained from the same animal, with the exception of the neck potentials in the 10-day row (asterisk), which were recorded in a 9-day rat Note shift of histograms towards high potential value which occurs earliest in A and latest in C Note also the general similarity in the shape of the histograms

birth and the 4th day there was only a small increase in potential On the 5th day, the potential had started to increase more rapidly and on the 10th day it had reached the adult level

The results of the mean membrane potential measurements thus indicate an increase in membrane potential which takes place between birth and the 10th day of life The increase is earliest in the neck muscles and latest in the gastrocnemius, the increase of the sartorius potential being intermediate (Fig 2 D)

Stable membrane potentials The scatter of the mean membrane potential values seen in Fig 2 might be due to the fact that the amplitude of the initial negative voltage deflection—instead of the more generally used sustained value—was chosen as the measure of the membrane potential Fig 3 illustrates the distribution of 16 stable membrane potentials recorded from the gastrocnemius muscle of an 8-day old rat It is seen that the stable membrane potentials do not differ markedly from the potentials of the 8-day gastrocnemius muscle in Fig 1

Mean maximum membrane potentials A major factor causing the shortcomings of the mean membrane potential measurements may be "faulty" penetrations which cause a proportion of the "true" membrane potential to be short-circuited (*cf* Boëthius and Knutsson 1970) In order to minimize this error, membrane potential was

Fig 2 Development of the mean membrane potential of the neck (A) sartorius (B) and gastrocnemius (C) muscles In the composite diagram (D) the developmental curves of the membrane potentials of the three muscles are shown together (symbols as in A—C) Note temporal difference in the membrane potential development of the three muscles Note also the scatter of the mean values indicating weakness in this method of estimating membrane potential development The lines approximating the potential development were fitted visually

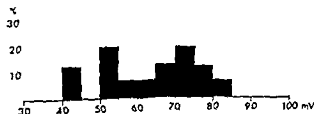


Fig 3 Distribution of sustained membrane potentials from the gastrocnemius muscle of an 8-day old rat Note the essential similarity to the histograms of Fig 1

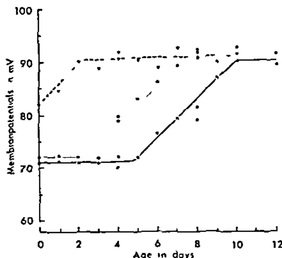


Fig 4 Development of the mean maximum membrane potential of the neck (broken line, triangles), sartorius (dotted line, circles) and gastrocnemius (solid line, squares) muscles Note temporal difference in potential development between the muscles Note also the similar rates of rise in potential of the muscles The leg muscle potentials show the developmental pattern of a plateau phase followed by a well defined "rise phase The lines approximating the development of the mean maximum membrane potentials were fitted visually

estimated by using the mean maximum membrane potential The results of these measurements are illustrated in Fig 4 At birth, the mean maximum potential of the neck muscle (82 mV) was *higher than those of the leg muscles During the next two* days it rose and reached a level of about 90 mV on the 2nd day Initially, the sartorius and gastrocnemius potentials were about 70 mV After the 2nd day the sartorius membrane potential started to rise, reaching the highest level (about 90 mV) on the 7th day The gastrocnemius maximum potentials started to increase after the 5th day and reached the highest level on the 10th day The relative difference in temporal order of development between the neck, sartorius and gastrocnemius muscles indicated by the mean membrane potential values was thus also shown by the mean maximum potentials Both in the sartorius and the gastrocnemius muscle the rise in maximum potential occurred during a period of 5 days Furthermore the rate of rise was the same both in the neck muscles and in the leg muscles

The distribution of the maximum membrane potential values was approximately normal The standard deviation varied between 2 and 4 mV and similarly to what was found for the mean values the scatter was about the same in all parts of the developmental curve The fact that the scatter did not increase during the period of increasing membrane potential may indicate that the time needed for the potential increase of the individual muscle cell is about 5 days If this time was appreciably shorter than the 5 day rise time of the mean value one would at least except to find an increased scatter on the ascending part of the developmental curve and possibly also a bimodal distribution

Action potentials In some experiments spontaneous action potentials were seen which appeared to be triggered by a fall in membrane potential caused by the microelectrode penetration Such action potentials were usually obtained when the dden negative potential shift of the initial cell penetration was followed by a

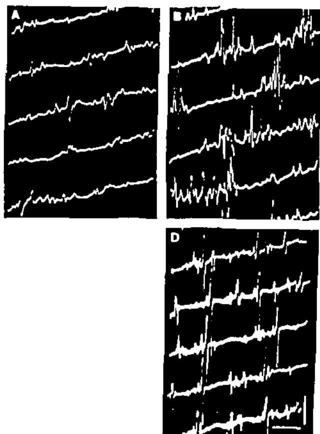


Fig 5 Electromyograms from a newborn (A gastrocnemius B neck muscle) and an 8 day (C gastrocnemius D neck muscle) rat Calibrations 50 μV 20 msec

towards the baseline The spike then took off from the declining resting potential An overshoot was practically never seen unless the initial negative potential shift was of the order of 70 mV Action potentials with a clear overshoot consistently had thresholds between 60 and 50 mV Abortive action potentials—without overshoot—could sometimes be seen also when the membrane potentials were considerably lower than 70 mV

Electromyograms EMGs were recorded in neck and gastrocnemius muscles of 10 rats between 0 and 10 days of age The records contained mono- bi tri and polyphasic potentials The polyphasic potentials were however not evaluated since it was usually impossible to determine unexceptionally that they derived from one motor unit During the first extrauterine days the motor unit potentials were often monophasic especially in the gastrocnemius muscle These potentials were less common in older rats In the youngest rats the motor unit potential amplitudes of the gastrocnemius muscles were smaller than those of the neck muscles *This difference* in amplitude disappeared after a few days Within the investigated period the duration of the motor unit potentials was longer in the gastrocnemius than in the neck muscle Both in the *gastrocnemius and the neck muscles there was a decrease in the*

duration of the motor unit potential during the 8 first postnatal days Fig 5 shows the general appearance of the EMG of the two muscles in a newborn and an 8 day rat When measured at higher magnification, the mean duration of the motor unit potentials of the newborn rat in Fig 5 A and B was found to be 5 3 msec for the gastrocnemius and 3 4 msec for the neck muscle In the 8 day rat of Fig 5 C and D, the corresponding values were 3 4 msec for the gastrocnemius and 2 1 msec for the neck muscle (Significance for the differences between muscles of the same kind and age tested with t tests, $p < 0\,01$) The results suggest a difference in functional maturation between motor units of the neck muscles and of the gastrocnemius muscle the motor units of the neck muscles being more advanced in their maturation

Muscle cell diameter The amplitude of the measured membrane potential may be influenced by the size of the penetrated cell (Haapanen Kolmodin and Skoglund 1958) It could therefore be argued that the increase in membrane potential during development was secondary to a growth in cell diameter To evaluate this possibility,

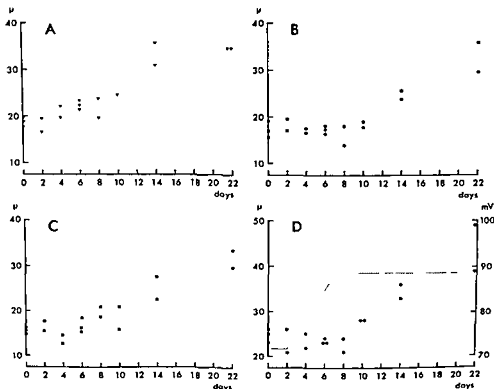


Fig 6 Mean diameters of muscle cells in neck (A) sartorius (B) and gastrocnemius (C) muscles Note the considerable scatter and the lack of unequivocal increase in mean diameter during the first 10 days The largest diameters of the sartorius muscles (circles) are shown in D together with the corresponding mean maximum membrane potentials (dotted line) Note lack of increase in diameter until after adult potential values are reached

muscle fibre diameters were investigated at different ages Each dot in the diagrams of Fig 6 A—C corresponds to the mean of 25 measurements from one muscle In each muscle the distribution of the measurements was approximately normal The standard error of the mean varied between 0 7 and 1 4 μ, and tended to be larger at higher ages The values obtained from the neck muscles were generally higher than the values from the sartorius and the gastrocnemius muscles The sartorius values also tended to be somewhat larger than those of the gastrocnemius

Fig 6 A—C shows that the scatter of the mean diameters was considerable in all age groups Furthermore, during the period when the membrane potential reaches adult values, *viz* 0—10 days, there was only an insignificant increase in diameter The results therefore do not support the contention that the increase in membrane potential is due to an increase in the mean muscle cell diameter

In the present study the membrane potentials and especially the maximum membrane potentials were probably recorded preferentially from the largest muscle cells Fig 6 D shows the development of the sartorius mean maximum membrane potential together with the diameters of the largest muscle cell in each investigated sartorius muscle It is seen that the rise in potential is completed before there is any increase in the largest muscle cell diameter Similar results were obtained for the neck and gastrocnemius muscles

Myotube myocyte transformation In a previous study, a temporal correlation was demonstrated between the rise in membrane potential and the transformation of myotubes into myocytes (Boethius and Knutsson 1970) In the present investigation an attempt was made to study this relationship more quantitatively Table I shows the percentage of myotubes of 30 cells in the neck sartorius and gastrocnemius muscles at different ages The results show that in the neck muscle the percentage of myotubes varies between 3 and 27 % at the time of birth After this time however only occasional myotubes are found The sartorius muscle starts out with about 20 % myotubes at birth but 2 days later the overwhelming majority of the cells are myocytes In the gastrocnemius muscle finally, the relative number of myotubes is about 20 % at birth Contrary to what is found for the sartorius however this high percentage of myotubes remains constant in the gastrocnemius muscle up to the 3rd day

Table I Percentage of myotubes (n = 30) in the neck sartorius and gastrocnemius muscles between birth and 5 days 3 rats (I II and III) were investigated at each age

	Neck				Sartorius				Gastrocnemius			
Age in days	I	II	III	X̄ ± SE	I	II	III	X̄ ± SE	I	II	III	X̄ ± SE
0	3	10	27	13 3 ± 7 1	27	27	23	25 7 ± 1 3	—	23	27	25 0 ± 2 0
1	0	—	0	0	0	20	7	9 0 ± 5 9	20	23	20	21 0 ± 1 0
2	3	0	0	1 0 ± 1 0	0	7	3	3 3 ± 2 0	23	17	20	20 0 ± 1 7
3	0	7	0	2 3 ± 2 3	0	3	0	1 0 ± 1 0	17	23	27	22 3 ± 2 9
4	—	—	—	—	—	0	0	0	10	—	—	10
5	0	0	0	0	0	0	3	1 0 ± 1 0	3	3	0	2 0 ± 1 0

After the 3rd day the number of myotubes decreases and on the 5th day the per centage of myotubes is insignificant

Effect of denervation It is known that denervation will retard and possibly also block the myotube myocyte transformation (Zelena 1962 Engel and Karpati 1968) Neonatally denervated rats were therefore investigated to obtain further evidence on the possibility of a functional coupling between the developmental rise in membrane potential and the myotube myocyte transformation

Fig 7 A shows a cross section from the denervated gastrocnemius of a 15 day old rat It can be seen that myotubes are fairly common The cells have a rounded appearance and the extracellular space is prominent The general impression is that of an embryonic muscle Fig 7 B shows a cross section from the contralateral un denervated gastrocnemius in the same rat In this muscle all nuclei are peripherally situated The polygonal shapes of the cells and the less prominent extracellular space are characteristic of the mature muscle In the four denervated rats examined in this study (sacrificed at 9 13 15 and 19 days) the percentage of myotubes in the de nervated gastrocnemius was about 20 % Up to the age of 19 days the percentage of myotubes in the denervated gastrocnemius thus remained at a level which in the normal gastrocnemius is otherwise found only up to the 3rd day

Membrane potentials were determined in the gastrocnemius muscles of the two oldest rats ar at 15 and 19 days The results are illustrated in Fig 8 The four histograms shown in this figure are similar the mean potential values being about 80 mV in all four muscles The difference between the mean values determined by a

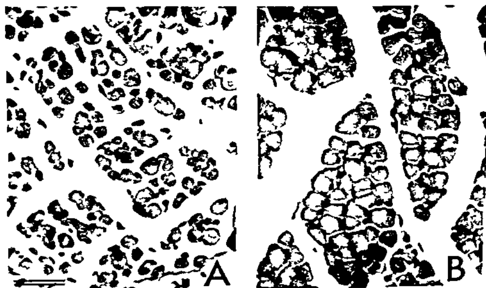


Fig 7 Cross-sect on of denervated and control gastrocnem us muscles of a 15-c muscle denervated at b rth re nnervat on had occurred Note great number large extracellular space B contralateral control muscle

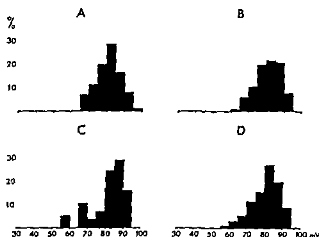


Fig 8 Distribution of membrane potentials in denervated and control gastrocnemius muscles A denervated muscle of 15 day rat (same as in Fig 7) B contralateral control muscle C denervated muscle of 19-day rat D contralateral control muscle Note adult membrane potential in all muscles

simple analysis of variance was not significant at the 10 % level (F = 0 27, df = 3/546) The mean maximum potential values of the four muscles were also of the same order of magnitude (about 90 mV) and showed no significant difference (P>10 %, F = 0 07 df = 3/30)

Reinnervation had occurred in all the denervated muscles as demonstrated by the fact that the denervated gastrocnemius and the contralateral control muscle gave twitches of similar magnitude after percutaneous stimulation of the lumbar plexus (The calcaneous tendon was connected to a strain gauge and the twitch displayed on an oscilloscope and photographed) In view of the denervation technique of the present study it is surprising that reinnervation occurs after such a short time since compression of the nerve brings the development of the postsynaptic membrane to a standstill for 2—3 weeks (Teravainen and Juntunen 1968) However since both the threshold and the strength duration curve were similar for the two muscles it is unlikely that the twitch obtained in the denervated gastrocnemius was caused by direct stimulation of the muscle

The results of the experiment on the denervated rats thus suggest that no causal relationship exists between the developmental rise in membrane potential and the myotube myocyte transformation Since the muscle was already reinnervated at the time of the experiments it is not possible to state whether the rise in membrane potential is dependent on the innervation or not

Discussion

Membrane potentials In the present work the development of the membrane potential was investigated by measuring the mean membrane potential and the mean maximum membrane potential Apart from the expected difference as to absolute potential values the results obtained with the two methods also differed in the following respect During a period of 5 days the maximum potential of the leg muscles rose up to an adult value In the mean potential measurements the duration of this

phase could not be ascertained unambiguously It is therefore pertinent to discuss which of the two types of measurements gives the best estimate of the true membrane potential Erroneously *high* potential recordings can be obtained by certain types of microelectrodes Because of the different composition of the intracellular fluid at different ages (see *e g* Vernadakis and Woodbury 1964), this error might vary with age With the type of electrodes used in this study, it seems unlikely that such an error has been very important Erroneously *low* values are on the other hand very common They are mainly caused by imperfect cell penetrations and large tip potentials Consequently, it seems reasonable to rely more on the high than on the low potential values and to regard the maximum potentials as the best estimate of the true membrane potential

In a preliminary study (Boethius 1969), the developmental pattern of the maximum potentials of the neck and gastrocnemius muscles corresponded to what was found in this study All absolute potential values were, however, about 10 mV higher in the present study This difference as to absolute values can probably be ascribed to the fact that the micropipettes of the two studies had somewhat different properties since they were pulled from different batches of tubing It was found in preliminary experiments that such a change could cause a difference in the general potential level even though the electrodes were otherwise very much similar In view of this methodological difficulty one should presumably rely more on the general developmental pattern than on the absolute magnitude of the measured membrane potential

In a study on the development of the mean membrane potential of the rat semimembranosus and gracilis muscles Fudel Osipova and Martynenko (1962) found this potential to be 23 mV at birth (see also Novikova 1964) From this low value the potential rose steadily and reached the adult value of 80 mV at the 15th day The rate of rise in potential was similar to that described in the present study The discrepancies may be due to the fact that Fudel Osipova and Martynenko used fairly large microelectrodes (resistance 6—10 Mohm) in their work

The membrane potential of the rat gastrocnemius muscle has been reported to increase between the 17th and the 24th day (Hazlewood and Nichols 1967) The measurements were made in excised and perfused legs and the mean potential values were 36 69 mV between 10 and 17 days and 67 76 mV between 24 and 27 days The corresponding standard deviations were 57 8 mV and 37 8 mV In another study on the gastrocnemius muscle of anesthetized rats Hazlewood and Nichols (1969) found a mean membrane potential of about 35 mV in a 8 day old rat the youngest animal in which any membrane potentials could be obtained After the 8th day the mean membrane potential rose exponentially and reached adult values of about 80 mV around the 40th day There is no obvious explanation for the discrepancy between the potential values reported by Hazlewood and Nichols (1967 1969) and the potentials of the present investigation It is interesting to note however that muscle electrolyte determinations performed by Hazlewood and Nichols (1969) showed a rapid decrease in intracellular sodium during the 10 first days after birth (see below)

In chick embryos the muscle membrane potential remains at a constant low level

*from a very early stage up to around the time of the myotube myocyte transforma*tion when there is a phase of rapid rise in potential (Boethius and Knutsson 1970) After four days the highest potentials have reached adult values and the rapid rise of the mean membrane potential levels off The development of the chick membrane potential thus follows a time course very similar to what is found for the potentials of the rat leg muscles in the present investigation Thus it seems reasonable to assume that a pattern characterized by an early plateau phase followed by a sharp rise phase is typical for membrane potential development in general Adopting this argument to explain the developmental curve of the neck muscle potential in this study, it follows that the middle of *the rise phase of the neck muscle potential co*incides with the time of birth

In chick embryos the rise in membrane potential commences shortly after the first appearance of the motor end plate (see Boethius and Knutsson 1970) In the gastrocnemius muscle of the rat the end plate can be visualized with the Koelle *Friedenwald* technique as early as 2—4 days *before birth (Csillik 1960)* The rise in the gastrocnemius membrane potential was found to occur as late as the 5th day after birth This difference between the chick and the rat as to the temporal relation between the rise in membrane potential and the end plate formation may suggest the lack of a simple functional coupling between the two developmental parameters *owever the presence of an end plate may yet be a prerequisite for a rise in the* mbrane potential It should also be mentioned that after denervation of adult rats the decrease in membrane potential (Thesleff 1963) and the rapid decrease in acetyl cholinesterase activity (Guth Albers and Brown 1964) occur roughly simultaneously

The fact that the rise in membrane potential occurs at different times in the three investigated muscles indicates that the rise is caused by processes generated in the muscle cells and not by some general factor such as the decrease in extracellular potassium (see *e g* Widdowson and McCance 1956 Skoglund 1967) or some hormonal effect In the early postnatal period there is a decrease in the intracellular sodium *concentration of the gastrocnemius muscle cells of the rat whereas the intracellular* potassium concentration remains roughly constant (Vernadakis and Woodbury 1964 Hazlewood and Nichols 1969) The change in sodium concentration seems to occur earlier in the neck than in the gastrocnemius muscle (Bergstrom Boethius and Hultman 1970) Therefore it seems reasonable to assume that the decrease in intracellular sodium concentration is related to the increase in membrane potential the underlying mechanism possibly being a change in the active transport of sodium or a decrease in the relative sodium permeability of the membrane

Electromyograms The results of the EMG recordings from neck and gastrocnemius muscles indicate that at birth the motor units of the neck muscle are functionally more mature than those of the gastrocnemius muscle and that there is a progressive maturation of the functional capacity of the motor units of both muscles during the first 8 days after birth

The finding that the motor unit potentials tended to have relatively long durations in very immature muscles agrees with similar findings in chick embryos (Boethius

1967) This long duration is probably due to a poor synchronization between the muscle fibres of the motor unit which may be caused by delays in the motor nerve terminals and at the end plate With increasing maturation of these latter elements the synchronization of the motor unit improves and reaches a stage comparable to that of the adult At this point the duration of the potential presumably reaches a minimum since investigations on humans (Buchthal Pinelli and Rosenfalck 1954) have shown that there is an increase in the duration of the motor unit potentials during later ontogeny

Morphology In the sartorius and the gastrocnemius muscles of the rat significant numbers of myotubes were found only up to the day when the rise in membrane potential started In the neck muscle on the other hand the rise in membrane potential and the myotube-myocyte transformation seem to occur more or less at the same time in a way reminiscent of what is found in the chick embryo (Boethius and Knutsson 1970) This variation in the temporal relation between the rise in potential and the myotube myocyte transformation in the same species suggests that these processes are not interdependent

In no instance was the percentage of myotubes of any muscle more than 27 % In the relatively most immature muscle the gastrocnemius the percentage of myotubes remained at about the same level from birth up to the 3rd day On the 4th day the percentage had decreased to 10 % and at 5 days to 2 % In the rat new muscle cells are known to be formed after birth (Morpurgo 1898) In view of this fact the roughly constant percentage of myotubes in the gastrocnemius muscle between birth and the 3rd day suggests a continuous contribution of newly formed myotubes which is balanced by a concomitant transformation of old myotubes into myocytes

Effects of denervation In normal development the rise in membrane potential takes place concomitantly with or immediately after the myotube myocyte transformation In denervated and subsequently reinnervated muscles of the present study the membrane potential rises to adult values despite the fact that about 20 % of the muscle cells are myotubes These results demonstrate that the developmental increase in membrane potential can occur independently of the myotube myocyte transformation

Qualitatively the present results on the effect of denervation on the myotube myocyte transformation confirms previous works of Zelena (1962) and Engel and Karpati (1968 The fact that the percentage of myotubes is about the same in the denervated gastrocnemius as in the gastrocnemius of the newborn rat suggests that the denervation may affect both the myotube-myocyte transformation and the development of new myotubes from primitive stem cells

[illegible] Physiology II from the Association [illegible] search Council Magnus Bergvalls [illegible] Medicinsk Forskning and Karo-

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The Effect of Deep Halothane Hypotension upon Labile Phosphates and upon Extra- and Intracellular Lactate and Pyruvate Concentrations *in the Rat Brain*

By

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Abstract

NILSSON L and B K SIESJO *The effect of deep halothane hypotension upon labile phosphates and upon extra- and intracellular lactate and pyruvate concentrations in the rat brain Acta physiol scand* 1971 *81* 508—516

A study was made of the energy metabolism of the rat brain during deliberate hypotension induced by 2 % halothane and partial airway obstruction A reduction of the mean arterial blood pressure to 30 mm Hg led to moderate increases in the extra and intracellular lactate and pyruvate concentrations and in the lactate/pyruvate ratios and to moderate decreases in the phosphocreatine content At the same blood pressure level hyperventilation with reduction of the P_aCO_2 to about 20 mm Hg aggravated the metabolic changes indicating a further reduction of the cerebral blood flow Although hypercapnia with an increase in the P_aCO_2 to about 85 mm Hg decreased the tissue lactate content markedly the oxidative metabolism was not normalized

It is concluded that although halothane hypotension with a reduction of the mean arterial blood pressure to about 30 mm Hg leads to changes in the energy metabolism of the brain the changes are so moderate that a critical degree of hypoxia probably can be excluded This conclusion may be even more valid if the mean blood pressure is not allowed to fall to such low values and if marked hyperventilation is avoided at very low blood pressures

Recently, a technique has been used clinically for inducing deliberate hypotension by using high halothane concentrations and an increased mean airway pressure (Ransohoff *et al* 1969) According to observations from several clinical centra the mean arterial blood pressure can be reduced to 30—40 mm Hg with this technique without creating signs of critical underperfusion of tissues or clinical complications

The potential value of the technique prompts an experimental investigation of its effects upon the energy metabolism of the brain Thus if the mean arterial blood pressure is reduced by bleeding to about 30 mm Hg in animals anesthetized with nitrous oxide marked metabolic changes will occur indicative of a relatively serious disturbance of energy production in the tissue (Kaasik *et al* 1970 *Siesjo and* Zwetnow 1970 a) A comparison with the clinical data suggests that the procedure

of inducing the hypotension with a high concentration of halothane protects the tissue from hypoxia but it is not known how far the blood pressure can be reduced without causing disturbances in the oxidative metabolism Moreover, deep halothane hypotension is frequently induced concomitant to a relatively marked reduction of the arterial CO tension This raises the question whether or not hyperventilation can reduce the cerebral blood flow during the hypotension to such a degree that metabolic changes will occur

The main objective of the present investigation was to study if halothane hypotension with a reduction of the mean arterial blood pressure to about 30 mm Hg will induce changes in the energy metabolism of the brain as reflected in the tissue contents of phosphocreatine, ATP, ADP and AMP, and in the extracellular and intracellular lactate and pyruvate concentrations The effects of hyperventilation and of hypercapnia were studied in other groups of animals maintained at a mean arterial blood pressure of 30 mm Hg

Methods

All experiments were performed on male Wistar rats weighing 300—400 g The animals were allowed free access to water and rat pellets (San Bolagen Malmo) Anesthesia was induced with divinyl ether in a closed jar When the animals were unresponsive to external stimuli they were taken from the jar immediately tracheotomized and connected to a respirator of the Starling type (Braun Melsungen) A gas mixture of 70 % N_2O and 30 % O was administered to the inlet of the respirator When the animals were removed from the jar tubocurarine chloride (Tubocurarin Vitrum) was injected i p in a dose of 0 5 mg/kg The respirator was preset so as to give an arterial CO_2 tension of 30—40 mm Hg If the pCO_2 was lower or higher than the values given the respirator was accordingly adjusted

One femoral artery was cannulated for blood pressure recording with an electromanometer (Elema Stockholm) and for anaerobic sampling of arterial blood In each experiment blood was also collected directly into liquid nitrogen for subsequent analyses of lactate and pyruvate The equipment used for measuring the blood pressure was frequently calibrated with a column of water The zero point used was the level of the heart Mean pressures were obtained by electrical integration The body temperature was measured in the rectum with a mercury thermometer The temperature was kept close to 37° C by means of intermittent heating from a lamp bulb

The atlantooccipital membrane was exposed for subsequent sampling of cisternal cere-

experiment the brain tissue was frozen by pouring liquid nitrogen into the funnel With this technique the animal was continuously ventilated during the freezing of the brain and the blood pressure did not drop until the freezing front reached the brain stem (see Granholm *et al* 1968 Nilsson and Siesjo 1970) The frozen brain was then chiselled out with cold instruments under intermittent irrigation with liquid nitrogen and the samples were stored at —80° C until analysed

Arterial blood was analysed for pH pCO_2 and pO_2 using microelectrodes operated at 37° C (Radiometer Copenhagen and Eschweiler & Co Kiel) and the values were corrected for temperature The pH values were referred to the phosphate buffers of the NBS (pH 6 841 and 7 383 at 37° C) In a few control experiments (see below) the CO_2 tension was measured in cisternal CSF using the micro CO_2 electrode Samples of arterial blood and cisternal CSF were analysed for lactate and pyruvate using specific enzymatic techniques The su

1 or 2 cm cuvettes Repeated blank samples were run in order to allow a correction for the AMP contamination of the NADH preparation used (Lowry *et al* 1964)

The concentrations of the metabolites measured were expressed in mmoles/kg of wet tissue In the case of lactate and pyruvate, intercellular concentrations were calculated (mmoles/kg of intercellular water) after corrections for the lactate and pyruvate contained in the blood and extracellular volumes of the tissue, which were assumed to be 3 and 12 %, respectively, of the tissue weight (see Kjallquist *et al* 1969)

When the animals were considered to be in a respiratory steady state on nitrous oxide, halothane was delivered to the respirator in a progressively increasing concentration until the dial setting of halothan vaporizer (Halothan-Verdunster, Dragerwerk, Lübeck) was 2 % The administration of nitrous oxide was discontinued when halothane was delivered

The halothane concentration was checked by a halothane analyser (Hook & Tucker, Ltd, Halothane Meter) for a given flow of gas through the vaporizer (see Wolfson 1968) The actual concentrations delivered by the particular halothan vaporizer used at each setting of the control dial were as given

The halothane output of the Halothan Verdunster used

Indicated dial reading (%)	Actual halothane output (%)
0 5	0 45
0 6'	0 60
0 7	0 70
2 0	1 90

rterial blood pressure sure was then upheld ory clamp In two of maintained but the % in one and 100 % with 2 % halothane in oxygen to an arterial CO_2 tension of about 20 mm Hg before the airway clamp was applied In the fourth group finally 7 % CO_2 was administered to the animals before the airway pressure was increased

A control group was obtained by administering 2 % halothane to the animals for 30 min *without airway obstruction However since this concentration of halothane gave rise to a fall* in blood pressure to 55—65 mm Hg the results were also compared to those obtained using 0 6 % halothane (Siesjo and Messeter 1971) This group was chosen for comparison instead of a previous one (Nilsson and Siesjo 1970) since it included analyses of the AMP contents

We have previously reported that when the mean arterial blood pressure was reduced to about 30 mm Hg by bleeding constantly ventilated rats during nitrous oxide anesthesia there was a marked fall in the arterial CO_2 tension indicating diminished CO_2 onutput from the tissues (Kaasik *et al* 1970) There was also a marked increase in the pCO_2 difference between the CSF and the arterial blood probably reflecting both a decreased cerebral blood flow and a reduced CO_2 transport capacity of the blood In the present experiments the reduction of the mean arterial blood pressure to 30 mm Hg did not give rise to any significant decrease in the arterial CO_2 tension or to any obvious increase in the CSF/arterial CO_2 tension difference Thus the CSF/arterial pCO_2 differences measured in three animals in the hyper ventilated group were 5 5 5 9 and 4 9 mm Hg respectively It was therefore concluded that the variations in the arterial CO_2 tension induced were accompanied by corresponding variations in tissue CO_2 tensions

Results

The results of the experiments are shown in Table I and II Table I gives the acid-base parameters of arterial blood as well as the lactate and pyruvate concentrations of whole blood and of cisternal CSF The table shows that as compared to the group of animals anesthetized with 0 6 % halothane those given 2 % halothane showed a decrease in the mean arterial blood pressure to 55—65 mm Hg This fall in blood pressure was associated with slight decreases in pH, in the actual plasma bicarbonate concentration and in the base excess value However, in spite

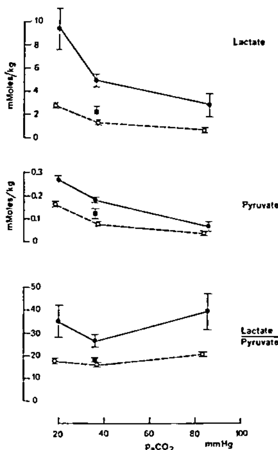


Fig 1 Lactate and pyruvate concentrations (mmoles/kg tissue) in rats anesthetized with 2 % halothane and subjected to a decrease in the mean arterial blood pressure to 30 mm Hg in normocapnia hypercapnia and hypocapnia (closed circles) The values have been compared to those obtained in a normotensive group anesthetized with 0 6 % halothane (open circles) as well as to a normocapnic group given 2 % halothane without an induced increase in the mean airway pressure (squares)

of the fact that there was a moderate increase in the lactate/pyruvate ratio there was no significant increase in the whole blood lactate content This was in contrast to CSF which showed a highly significant increase in both the lactate concentration ($p < 0\,001$) and in the lactate/pyruvate ratio ($0\,001 < p < 0\,01$)

When the mean blood pressure was decreased to 30 mm Hg by means of an increased mean airway pressure a significant lactacidosis occurred in the blood and there was a pronounced increase in the lactate/pyruvate ratio In this situation there was a less marked increase in the CSF lactate concentration and no significant further changes in the lactate/pyruvate ratio The results did not indicate any significant differences between the groups given 30 and 98 % oxygen respectively

When the arterial CO tension was reduced to a mean value of about 20 mm Hg the arterial plasma pH normalized The hyperventilation did not seem to aggravate the lactate accumulation In the CSF however there was an increase in the lactate and pyruvate concentrations but no significant change in the lactate/pyruvate ratio

In the group exposed to 7 % CO before the airway obstruction there were

[illegible]

Exp group	Arterial blood P_aO_2	P_aCO_2	pH	(HCO_3^-)	BE
Halothane 0 6 % MABP = 140 mm Hg (n = 4)	145 ±15	37 9 ±0 9	7 435 ±0 012	24 7 ±0 6	1 3 ± 0 8
Halothane 2 % MABP = 55—65 (n = 5)	260 ±13	36 3 ±0 8	7 398 ±0 014	21 6 ±0 60	— 1 8 ± 0 8
Halothane 2 % MABP = 30 O_2 = 30 % (n = 6)	149 ± 6	34 7 ±1 6	7 281 ±0 031	15 6 ±1 0	— 9 5 ± 1 5
Halothane 2 % MABP = 30 O_2 = 98 % (n = 4)	260 ± 7	36 0 ±1 1	7 307 ±0 026	18 0 ±1 2	— 7 5 ± 2 1
Halothane 2 % MABP = 30 O_2 = 98 % (n = 7)	314 ± 8	19 7 ±0 9	7 390 ±0 032	11 7 ±0 8	—10 9 ± 1 4
Halothane 2 % MABP = 30 CO_2 = 7 % (n = 4)	333 ±11	85 0 ±0 8	6 966 ±0 008	18 0 ±0 3	— 16 1 ± 0 4

pronounced decreases in the arterial pH and in the base excess values In arterial blood there was only a moderate reduction of the lactate concentration and no significant change in the lactate/pyruvate ratio In the CSF, however, the lactate concentration was reduced to a third of that measured during hyperventilation In spite of the large changes in the lactate concentrations the lactate/pyruvate ratios were rather similar in all groups in which the mean blood pressure had been reduced to 30 mm Hg

Table II lists the tissue metabolites measured in the groups of Table I The figures show that the blood pressure drop accompanying the administration of 2 % halothane was unassociated with any significant changes in the tissue contents of phosphocreatine ATP ADP or AMP There were moderate increases in the lactate and pyruvate contents and in the lactate/pyruvate ratio However it was found that the increase in the mean lactate content was due to relatively large increases in two of the animals (3 17 and 3 69 mMoles/kg respectively) Both these animals had mean arterial blood pressures of 55 mm Hg In the remaining 3 animals which had blood pressures exceeding 60 mm Hg the tissue lactate contents were similar to the control values Thus as long as the mean blood pressure exceeded a value of about 60 mm Hg there were no detectable metabolic changes

When the airway was partially obstructed so as to induce a fall in the mean arterial

blood and cisternal cerebrospinal fluid in rats anesthetized with 2 % halothane The experimental (Siesjo and Messeter 1971) The administration of 2 % halothane led to a fall in mean arterial deliberately reduced to 30 mm Hg by means of a partial airway obstruction The last two groups CO_2 tensions are given in mm Hg The bicarbonate concentrations and the base excess values in

			CSF		
La	Py	La/Py	La	Py	La/Py
1 82	0 147	12 6	2 40	0 170	14 3
±0 13	±0 007	±1 1	±0 11	±0 013	±0 6
1 94	0 121	15 6	4 80	0 232	21 4
±0 42	±0 018	±1 2	±0 37	±0 025	±1 9
8 29	0 202	43 7	6 25	0 265	23 6
±1 0	±0 020	±7 7	±0 52	±0 018	±1 4
8 12	0 174	46 9	6 02	0 248	24 1
±0 55	±0 004	±3 5	±0 73	±0 015	±1 6
9 15	0 186	54 2	9 29	0 337	27 0
±0 92	±0 024	±7 8	±1 30	±0 033	±1 5
5 96	0 118	52 3	3 17	0 114	27 8
±0 42	±0 012	±7 0	±0 14	±0 006	±1 8

blood pressure to 30 mm Hg there were marked increases in the lactate and pyruvate contents and in the lactate/pyruvate ratios These changes were accompanied by moderate decreases in the phosphocreatine contents (see Discussion) in some but not in all animals but unaccompained by any changes in ATP ADP or AMP There were no differences between the groups given 30 and 98 % oxygen respectively

In the hyperventilated group there was a further pronounced increase in lactate and pyruvate as well as in the lactate/pyruvate ratio and a more marked fall in the phosphocreatine content It should also be remarked that this group showed the highest AMP values

In the group given 7 % CO_2 there was a moderate increase in the lactate content and a marked increase in the lactace/pyruvate ratio (see below) The low phosphocreatine value is misleading if not compared to that obtained in hypercapnia of a comparable degree Thus pure hypercapnia during halothane anesthesia will by itself lower the phosphocreatine content of the tissue to about 4 3 mmoles/kg (Siesjo and Masseter 1971)

The changes observed in the lactate and pyruvate contents of the tissue are better evaluated if compared to values measured at the same CO_2 tensions under halothane anesthesia Fig 1 compares the present values with those obtained in a recent study

TABLE II Brain tissue concentrations of phosphocreatine ATP, ADP, AMP, lactate and pyruvate (mMoles/kg of tissue) for the same groups of animals as shown in Table I Means ± S E

	BRAIN TISSUE							
Exp group	P_aCO_2	PCr	ATP	ADP	AMP	La	Py	La/Py
Halothane 0 6 % MABP = 110 mm Hg (n = 4)	37 9 ±0 9	5 13 ±0 16	2 82 ±0 04	0 34 ±0 01	0 030 ±0 003	1 28 ±0 07	0 081 ±0 003	15 9 ±0 9
Halothane 2 % MABP = 55—65 (n = 5)	36 3 ±0 82	5 28 ±0 10	2 93 ±0 02	0 36 ±0 01	0 021 ±0 004	2 22 ±0 50	0 121 ±0 024	18 1 ±0 8
Halothane 2 % MABP = 25—35 O_2 = 30 % (n = 6)	34 7 ±1 6	4 62 ±0 23	2 92 ±0 07	0 36 ±0 01	0 028 ±0 006	5 34 ±1 47	0 178 ±0 029	27 3 ±4 1
Halothane 2 % MABP = 25—35 O_2 = 98 % (n = 4)	36 0 ±1 1	4 85 ±0 35	2 89 ±0 03	0 38 ±0 02	0 028 ±0 005	4 86 ±0 56	0 183 ±0 013	26 6 ±3 0
Halothane 2 % MABP = 25—35 O_2 = 98 % (n = 7)	19 7 ±0 9	4 25 ±0 38	2 79 ±0 06	0 38 ±0 03	0 038 ±0 005	9 40 ±1 8	0 264 ±0 017	35 3 ±6 8
Halothane 2 % MABP 30 CO_2 = 7 % (n = 4)	85 0 ±0 8	4 11 ±0 46	2 93 ±0 03	0 36 ±0 03	0 022 ±0 004	2 89 ±1 1	0 069 ±0 016	39 7 ±8 4

in animals anesthetized with 0 6 % halothane (Siesjo and Messeter 1971) The figure shows that at any given CO_2 tension the lactate concentrations and the lactate/pyruvate ratios were higher in the hypotensive animals than in the normotensive ones

Discussion

The present results have shown that when the mean blood pressure falls in rats anesthetized with 2 % halothane there were no metabolic changes in the brain indicating a derangement of the oxidative metabolism until the pressure fell below about 60 mm Hg When the pressure was made to fall to about 30 mm Hg by means of an induced increase in the airway pressure, there were moderate metabolic changes These changes consisted of moderate increases in the intra and extra cellular lactate and pyruvate concentrations and in the corresponding lactate/pyruvate ratios, and possibly of a small decrease in the phosphocreatine content of the tissue When rats hyperventilated to a CO_2 tension of about 20 mm Hg were subjected to the same degree of hypotension the lactate and pyruvate changes were exaggerated and there was a significant fall in phosphocreatine, indicating a disturbed energy production in the tissue

The combination of increased lactate concentrations, increased lactate/pyruvate

ratios, and decreased phosphocreatine contents indicates that when the halothane hypotension is carried so far that the blood pressure falls to 30 mm Hg moderate cerebral hypoxia is present However, the changes observed were so discrete that it is questionable if the hypotension represented a threat to the viability of the cells Thus since a fall in intracellular pH induced by the increased lactate con centrations may have contributed to the fall in the phosphocreatine content (effect of pH on the creatine phosphokinase equilibrium, see Nilsson and Siesjö 1971, Siesjö and Messeter 1971) the hypotensive condition was associated with only a very small effect on the high energy stores of the tissue The tissue changes obtained during the halothane hypotension should be compared to the much more marked changes observed in nitrous oxide anesthetized animals bled to the same mean arterial blood pressures (Kaasik *et al* 1970) The comparison indicates that the absence of hypovolemia and/or the presence of high concentrations of halothane protect the tissue from the noxius effects of a low perfusion pressure

There was a marked variability in the metabolic response in the individual animal, changes observed in nitrous oxide — anesthetized animals bled to the same mean arterial blood pressure This indicates that a pressure of 30 mm Hg represents a critical threshald for the tissue perfusion (*cf* Siesjo and Zwetnow 1970 a and b, Zwetnow 1970), and that very small metabolic changes may be expected to occur at higher mean blood pressures It is noteworthy, though, that hyperventilation to an arterial CO tension of 20 mm Hg seems to aggravate the metabolic changes This finding indicates by itself that hypocapnia can reduce the cerebral blood flow in spite of the hypotension and the deep halothane anesthesia, an assumption which has been verified in cat experiments (Nilsson to be published) If the experimental results can be transferred to the clinical situation, it would thus seem advisable to avoid marked hyperventilation if the mean arterial blood pressure is allowed to fall to very low values

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Increase in Capillary Filtration Rate Resulting from Reduction in the Intravascular Calcium Ion-Concentration

By

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Abstract

NICOLAYSEN, G *Increase in capillary filtration rate resulting from reduction in the intravascular calcium ion-concentration* Acta physiol scand 1971 *81* 517—527

Marked reductions in intravascular concentrations of Ca^{++} and Mg^{++} has previously been shown to cause edema development The effect of moderate reductions in the intra vascular concentration of Ca^{++} on the net rate of filtration from a vascular bed during increased capillary pressure has been analyzed Isolated, ventilated rabbit lungs were perfused with plasma at 28° C In each preparation repeated filtration tests were performed by standardized increases in the outflow pressure lasting for 6 or 10 min The rate of weight increase for the preparation when 4 min of a test had elapsed was taken as the net rate of filtration This net rate of filtration was shown to be increased when the $[Ca^{++}]$ was reduced to about 0 1 mM This increase in filtration rate could be reversed by Ca^{++}-addition, but not by Mg^{++} addition Evidence is presented that this increase in filtration rate was due to changes in the 'hydraulic conductivity' of the capillary wall

The mechanisms and pathways for the movement of molecules across the capillary wall are by no means fully understood Ultrastructural research on capillary walls indicates that the clefts between the endothelial cells are the pathways for some large molecules (Karnovsky 1967) Pappenheimer (1953) proposed that these clefts were the main pathway also for water and smaller solute molecules On the basis of indicator-diffusion experiments Yudilevich and Alvarez (1967) and Alvarez and Yudilevich (1969) proposed, however, that water exchange to a considerable extent takes place *through* the endothelial cells If this were correct then the capillary permeability to water could theoretically be affected in two ways by change in the permeability of the endothelial cells to water or by changes of the intercellular clefts

Edema formation at physiological intravascular pressures is usually interpreted as indicating that the permeability of the capillaries to proteins has been markedly increased Such increases in permeability can be induced in various ways, e g by reducing perfusate $[Ca^{++}]$ and $[Mg^{++}]$ to very low levels (Nicolaysen 1971) Increase in capillary permeability to proteins of such a magnitude that edema develops must be regarded as a rather extreme change in permeability Surprisingly enough situations with more moderate changes in capillary permeability have not been conv demonstrated

On the basis of these considerations it was decided to determine whether smaller reductions in intravascular [Ca^{++}] and/or [Mg^{++}] than those resulting in edema formation affect the permeability of capillaries

Experiments were performed on perfused and ventilated rabbit lungs Changes in capillary permeability were observed from measurements of changes in net rate of filtration from the vascular bed Filtration was induced by increasing the outflow pressure from the preparation The term "hydraulic conductivity of the capillaries" (Pappenheimer 1953) will be used for the filtration-related "type" of capillary permeability that is investigated in the present experiments

A preliminary report on some of these experiments has been given earlier (Nicolaysen 1969)

Methods

removed

250 to 320 ml/min but it was kept constant throughout one experiment The perfusate volume was 200 ml at the outset of the experiments The lungs were *ventilated* with a 5 % CO_2 in air mixture, using a positive pressure ventilation technique In some experiments the CO_2 content was gradually reduced in order to keep the pH of the perfusate which was measured intermittently, within the physiological range The *pulmonary arterial pressure* was continuously recorded *Weight changes* of the preparation were continuously recorded by having the preparation suspended underneath a force transducer (Sanborn FTA 100 1) The lower point of the lungs was about 6 cm below the left atrium and the vertical extension of the preparation about 8 cm The first 20—40 ml of outflow from the preparation were discarded to avoid contamination of the perfusate with blood remaining in the vascular bed About 10 min after start of perfusion 7 5 mg papaverine sulfate in 1 5 cc of saline was added to the perfusate This was done to avoid changes in vascular resistance during the perfusion and to achieve an even perfusion of the pulmonary vascular bed

Capillary filtration tests (Pappenheimer and Soto-Rivera 1948 Mellander 1960) were performed by increasing the left atrial pressure (L A p by 10 mm Hg for 6 or 10 min After the filtration period the L A p was reduced to the prefiltration value of 0 5 1 mm Hg

The slope of the curve for the weight increase (expressed as g/min after the first 4 min of the filtration period had elapsed was taken as *the filtration rate* The first filtration test was performed 30 min after the start of perfusion The subsequent tests were applied at 40 min intervals At the end of such a 40 min period the weight of the preparation had in most experiments declined to a level about equal to that seen before the previous filtration test

Two *types of perfusates* were used

plasma portion was thawed at 38 C and then filtered once through one layer of filter paper

2) *Homologous rabbit whole blood* obtained by heart puncture of anesthetized (20—30 mg/kg

and heparinized (750 I U kg intravenously rabbits Two thousand

5 C One perfusion with rabbit whole

and of *total magnesium* [Mg_t]

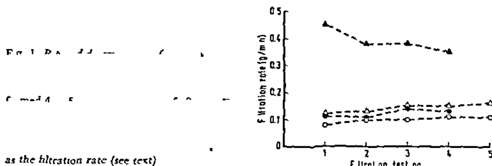


Fig 1 ... as the filtration rate (see text)

The concentrations of Ca++ and/or Mg++ ...

publication (Nicolaysen 1971)

Results

Filtration tests performed during perfusions with normal horse plasma (Group 1 experiments) To determine the stability of the perparation, sequences of filtration tests with unaltered perfusate were performed Fig 1 shows the results from four such experiments The filtration rate varied considerably from one preparation to another, but there was little variation from one test to another in the same preparation

The filtration rate at an EDTA concentration equal to the total plasma calcium concentration (Group 2 experiments) The concentration of total calcium in the horse plasma used was 3 0 mM About half of this calcium is presumably ionized A reduction in $[Ca^{++}]$ to about 0 1 mM by EDTA addition (concentration of complexed plus free EDTA = $[EDTA_t] = [Ca_t]$) is known not to cause edema formation in this preparation even if this low concentration is maintained for 2 h (Nicolaysen 1971) The capillary filtration rate at this degree of reduction in $[Ca^{++}]$ was compared to the filtration rate at normal $[Ca^{++}]$ The $[Mg^{++}]$ was slightly reduced at this EDTA concentration (Table I)

TABLE I Calculated concentrations of Ca^{++} and Mg^{++} in the plasma perfusates at different concentrations of chelators

Concentration of chelator	1 75mM EDTA	3 0mM EDTA	3 0mM EGTA	3 0mM EGTA with Mg added
Concentration of total calcium	3 0mM	3 0mM	3 0mM	3 0mM
Concentration of total magnesium	0 5mM	0 5mM	0 5mM	3 5mM
Calculated $[Ca^{++}]$	1 25mM	1 0 10^{-} mM	1 10 ⁻⁴mM	1 10^{-4}mM
Calculated $[Mg^{++}]$	0 5mM	0 4mM	0 5mM	3 5mM

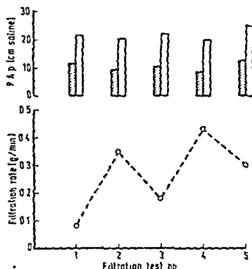


Fig 2 *Changes in capillary filtration rate induced by consecutive additions of EDTA and $CaCl_2$ to a plasma perfusate* Preparation as in Fig 1 Flow 285 ml/min The filtration rate was determined as in Fig 1 in 5 successive tests Five min after test no 1 and test no 3 an amount EDTA equimolar to the total calcium present in the perfusate at the outset of the experiment was added *Five min after tests no 2 and no 4* $CaCl_2$ *was added* in an amount equimolar to the previously added EDTA *In the upper part of the figure is shown* the pulmonary arterial pressure ($\bar{P}$ *A p*) 1 min before the start of a test (cross hatched bars) and the same pressure 4 min after start of the 6 *min test period* (open bars)

Fig 2 shows the results of one out of five similar experiments Five min after the end of the first capillary *filtration test 0 6* mmole of *EDTA (67 mM solution in saline)* was added to the perfusate Five min after the second filtration test 0 6 mmole of $CaCl_2$ (0 1 M in saline) was added After the third filtration test 0 6 mmole of EDTA as again added, and so forth Thus every second filtration test was done at nearly normal Ca^{++} and Mg^{++} concentrations and every second one at reduced concentrations of Ca^{++} and Mg^{++} In order to obtain a measure for alterations in filtration rate a *filtration ratio* was calculated This was obtained by dividing the value for each individual filtration rate by the mean of the values obtained in the immediately preceding and the immediately following test Filtration ratios from the Group 2 experiments are listed in Table II For six out of seven tests performed at reduced [Ca^{++}] and [Mg^{++}] the filtration ratios were considerably higher than 1 This could indicate

TABLE II *The effect on the lung capillary filtration rate of different degrees of reduction in perfusate*

	Group 3 experiments		Group 2 experiments					Group 4 experiments		
Presumed [Ca^{++}]	1 25mM ([$EDTA_t$] ≈ 0 6 [Ca_t])		1 10^{-1}mM ([$EDTA_t$] = [Ca_t])					1 10^{-4}mM ([$EGTA_t$] = [Ca_t])		
Experiment no	1	2	1	2	3	4	5	1	2	3
Filtration ratio	1 0	0 9	1 8	1 9	2 7	1 6	1 0	1 5	1 5	1 3
	0 9	1 4			1 8		1 4	1 4	1 5	1 8

[Ca_t] = concentration of ionized + non ionized calcium (3 mM) [$EDTA_t$] and [$EGTA_t$] = concentration of complexed + free chelator

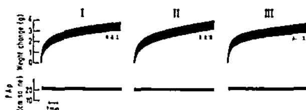


Fig 3 *Capillary filtration tests of 10 min duration at normal and reduced concentrations of Ca++ and Mg++* Preparation as in Fig 1 The 1st and the 3rd test were performed at normal plasma concentrations of Ca++ and Mg++, the 2nd at reduced concentration of these ions achieved by EDTA addition The pulmonary arterial pressure (P A p) is shown in the lower tracing The records start about 10 sec after the beginning of each test, which was initiated by increasing the left atrial pressure by 10 mm Hg R = filtration rate = weight increase per min in g

that this degree of reduction in [Ca++] and/or [Mg++] affected the hydraulic conductivity of the capillaries

An increase in vascular volume, and hence in preparation weight takes place when the intravascular pressures are increased It was important to know therefore, how long after an increase in left atrial pressure the intravascular volume went on increasing In one of the experiments of Group 2 the filtration periods were extended to 10 min In that experiment (Fig 3) 3 successive filtration tests were done, the second one after an EDTA addition which gave $[EDTA_t] = [Ca_t]$ the third after an appropriate $CaCl_2$ addition In every test the slope of the weight curve remained nearly constant from the 4th to the 10th min This linearity of the weight slope was taken to indicate that no increase of significance in intravascular volume took place after the 4th min (see Discussion)

In some of the experiments there was a tendency towards moderately lower perfusion pressures, and hence reduced total vascular resistance (the flow was maintained constant) during low [Ca++] conditions (Fig 2) The pulmonary arterial pressure (P A p) at reduced [Ca++] and [Mg++] was in no experiment lowered by more than 20 %, usually the pressure reduction was less than 10 % The experiment of Fig 4

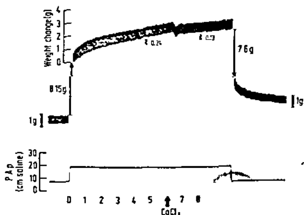


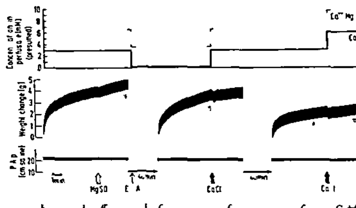
Fig 4 *The immediate effect on the filtration rate of increasing the intravascular concentrations of Ca++ from a reduced level* Preparation as in Fig 1 Flow 300 ml min 35 min before the filtration test EDTA was added to the plasma to give [EDTA] = [Ca] (thereby reducing [Ca++] to 0 1 mM) At time 0 the left atrial pressure was increased from 1 mm Hg to 11 When 6 min of the filtration test had elapsed $CaCl_2$ was added to the perfusate in an amount equal in mmoles to the EDTA previously added R = filtration rate = weight increase per min in g

shows that changes in filtration rate induced by changes in $[Ca^{++}]$ and $[Mg^{++}]$ can take place independently of changes in total vascular resistance Thirty five min before the test shown, EDTA was added to give $[EDTA_t] = [Ca_t]$ The weight increase was linear from the 4th to the 6th min of the test At this point $CaCl_2$ was added From the 7th to the 10th min the weight again increased linearly The slope of the curve was, however, reduced from 0 24 to 0 13 g/min There was no measureable change in P A p during that test

The filtration rate at $[EDTA_t] = 1/2\ ([Ca_t]+[Mg_t])$ *(Group 3 experiments)* Since the filtration rate was markedly increased at $[EDTA_t] = [Ca_t]$ (= 85 % of $([Ca_t]+[Mg_t])$) it was of interest to test the effects of lower concentrations of EDTA In two such experiments capillary filtration tests were performed as in the experiments of Group 2 The amount of EDTA added was adjusted to give $[EDTA_t] = 1/2\ ([Ca_t]+[Mg_t])$ At this EDTA level the estimated $[Ca^{++}]$ was 1 5 mM whereas the $[Mg^{++}]$ was unchanged (Table I) The filtration ratios at this $[Ca^{++}]$ level were calculated and are listed in Table II Only one out of four such filtration ratios was larger than unity

Relative role of reduction in Ca^{++} *and* Mg^{++} *in causing the increased filtration rate (Group 4 experiments)* At the EDTA-concentration used in the Group 2 experiments the $[Ca^{++}]$ and the $[Mg^{++}]$ were both reduced The use of the chelating agent ethyleneglycodiaminetetraacetic acid (EGTA) offered some possibility of differentiating between the roles of $[Ca^{++}]$ and $[Mg^{++}]$

When in one experiment an amount of EGTA equimolar to the perfusate content of $[Ca_t]$ was added to the reservoir, the filtration ratio was increased (Table II experiment 1 of Group 4) At this EGTA-concentration there was practically no reduction in the $[Mg^{++}]$ indicating that the reduction in $[Ca^{++}]$ was the critical parameter The possibility remained, however, that Ca^{++} and Mg^{++} were interchangeable in maintaining normal hydraulic conductivity of the capillaries and thus that the $([Ca^{++}]$ plus $[Mg^{++}])$ could be the important parameter Fig 5 shows the results of an experiment designed to clarify this problem Three 10 min filtration tests were performed in succession When six min of the first filtration test had elapsed, a $MgSO_4$ solution was added, containing as many mmoles of Mg as there were mmoles of calcium (total) in the perfusate The filtration rate was unaffected by this addition Five min after the end of this first filtration test EGTA was added in an amount equimolar to the $[Ca_t]$ This EGTA addition reduced the $[Ca^{++}]$ to a low level (1 10 ³ mM, Table II) but reduced the $([Ca^{++}]+[Mg^{++}])$ only to a normal level A second filtration test performed 35 min later, showed a higher filtration rate than in the first test When six min of this test had elapsed $CaCl_2$ (equimolar to the added EGTA) was added The filtration rate was reduced by this addition, which brought the $[Ca^{++}]$ up to a normal value and the $([Ca^{++}]+[Mg^{++}])$ up to the increased level achieved during the second part of the first filtration test Since an increase above the normal level of $([Ca^{++}]+[Mg^{++}])$ does not affect the filtration rate (first test) the effect of the $CaCl_2$ addition during the second test must have been due to the increased $[Ca^{++}]$ alone It can therefore be deduced that the effect of the added EGTA was due to the induced change in $[Ca^{++}]$ alone After the second filtration test there followed a 40 min period



per m n in g

of perfusion at low L A p and unaltered [Ca^{++}] and [Mg^{++}] in the perfusate A third filtration test was then performed After 6 min of filtration the same amount of $CaCl_2$ as before was added The filtration rate was not affected by this addition (Fig 5)

Two other experiments of slightly different design confirmed that Mg^{++} in the concentrations tested could not substitute for Ca^{++} in keeping the filtration ratio low The experimental procedure of the first experiments of Group 2 was followed Before the first filtration test was carried out as many mmoles of $MgSO_4$ were added as there were mmoles of calcium (total) in the plasma perfusate After the first test EGTA was added to give [$EGTA_t$] — [Ca_t] After the second test $CaCl_2$ was added and so forth The filtration ratios at tests with reduced [Ca^{++}] were significantly above 1 (Table II, Experiment no 2 and 3 of Group 4) The ([Ca^{++}] plus [Mg^{++}]) was however, then normal From the experiment shown in Fig 5 and one other experiment it was known that an increase in [Mg^{++}] or [Ca^{++}] above the normal value did not affect the filtration rate

Changes in filtration rate induced during whole blood perfusion In one experiment rabbit whole blood was used as a perfusate Three 6 min filtration tests were performed After the first test an amount of EDTA equimolar to the [Ca_t] in the plasma fraction of the perfusate was added After the second test CaCl was added The relative filtration ratio of the second test was calculated to be 1 4

Discussion

The results of the experiments are interpreted as showing that large enough reductions in intravascular concentration of calcium ions result in an increase in

draulic conductivity of the exchange vessels Changes in the protein permeability were, if present, not large enough to cause edema formation The first of these conclusions is based on observed changes in the rate of the weight increase during periods of elevated intravascular pressures (filtration tests) In general the weight increase of the lung preparation during a filtration test may be due to 1) Increase in intravascular volume in the weighed preparation, 2) Net outward flux of fluid from the exchange vessels (filtration)

An increase in intravascular volume obviously occurred in response to the increased transmural pressure of the vessels (and of the left atrium and auricle) (Fig 4) However, this increase does probably mainly take place in the first few min of the test The weight increased linearly after the first four min of a test had elapsed, and at the same time the left atrial pressure and the pulmonary arterial pressure were constant (Fig 3) A linear augmentation in intravascular volume at unchanged intravascular pressures and for as long periods as 6 min (Fig 3) is improbable A linearly increasing weight could theoretically be explained as a result of a concomitantly decreasing rate in volume augmentation and an increase in the rate of filtration Since the filtration rate is most probably constant or slightly decreasing through one such test (constant pressure head) it is reasonable to presume that no significant increase in vascular volume takes place after four min of a test has elapsed

The rapid and transient deviations from the linear slope of the weight curve seen in some of the experiments (Figs 4 and 5) in response to a change in [Ca^{++}] and/or [Mg^{++}] during a filtration test were probably due to transient changes in vascular volume Within one min of the addition the rate of weight increase again became linear but not necessarily with the same slope as before

On the basis of these findings and considerations it seems justified to conclude that the slope of the linear parts of the weight curves during filtration tests can be used as a measure of net filtration rate from the exchange vessels This filtration rate depends on several factors 1 Total surface area of exchange vessels through which outward filtration takes place during the test 2 The filtration pressure in these vessels 3 The hydraulic conductivity of these vessels

In this preparation the total area through which filtration can take place is unknown and cannot easily be measured Changes in this area can take place through a change in number of or diameter of perfused exchange vessels (capillaries) In some of the experiments the pulmonary vascular resistance decreased (Fig 2), although to a small extent when the concentrations of calcium and magnesium ions in the perfusate were reduced This could imply that the area available for filtration increased The experiments illustrated in Fig 4 and 5 show however that the changes in filtration rate can occur independently of measurable changes in total pulmonary vascular resistance It is generally assumed that in the rather specialized vascular bed of the lung, the resistance to flow through the capillaries accounts for a large fraction of the total vascular resistance A change in number of or in size of perfused capillaries should thus cause some measurable change in vascular resistance These arguments, together with the point that it is unlikely that large changes in number

of perfused capillaries should be induced by changes in intravascular concentration of calcium and magnesium ions in this papaverinized preparation, seem to justify the conclusion that the observed changes in filtration rate were not to any extent due to changes in surface area of perfused capillaries. Since there is no reason to presume that changes in filtration pressure can explain the results, only changes in hydraulic conductivity of the capillaries remains as an explanation.

The effects of addition to the perfusates of the chelators are presumed to be due to the reductions induced in concentration of calcium and magnesium ions (Nicolaysen 1971). In the present experiments the effect of addition of chelators was shown to be due specifically to reduction in perfusate concentration of calcium ions. High intravascular concentrations of magnesium ions could not counteract the effects of low concentrations of calcium ions on the filtration rate. It has previously been shown (Nicolaysen 1971) that a far more extensive reduction in concentration of calcium ions than those imposed in the present experiments does not cause edema formation of the preparation unless the concentration of magnesium ions also is very low. A tempting explanation of these observations is that the water transport across the capillary wall can take place via two different routes, one which is a common one with large molecules, and one through which these large molecules cannot pass. Reductions in intravascular concentration of calcium ions could then possibly affect the latter route only, whereas a reduction in both $[Ca^{++}]$ and $[Mg^{++}]$ (or $[Mg^{++}]$ alone) affects the common pathway. The findings could thus support the hypothesis of Alvarez and Yudilevich (1969). The present results can, however, also be explained on the basis of only one pathway which by isolated reduction in $[Ca^{++}]$ is somewhat altered, but not to such an extent that the protein permeability is much increased.

One interesting point is whether the capillary permeability to small molecules other than water is increased at the conditions of increased hydraulic conductivity. The present experiments do not allow conclusions on this point, but further tests are presently being carried out.

The present experiments with their repeated filtration tests, could be carried out only because the effects of reduced concentrations of calcium ions were reversible. The experiments of Fig. 4 and Fig. 5 illustrate the very short latency of the reversal of the effects caused by low concentration of calcium ions. Reductions in the rate of filtration were apparent within one min from the point of $CaCl_2$ addition. The development of EDTA- induced edema in this preparation can also be stopped at early stages by $CaCl_2$ addition. Also there the effect had the same short latency (Nicolaysen 1971).

The changes in filtration rates observed at $[EDTA_t] = [Ca_t]$ in the plasmas varied from one experiment to another (Table II). The changes in filtration rate in the EGTA experiments ($[EGTA_t] = [Ca_t]$) were no larger than in the EDTA experiments (Group 2), although the $[Ca^{++}]$ (from calculations) could be ten times higher in the latter group (Table I). Since one would presume that the change in filtration rate should in some way be dependent on the degree of reduction in $[Ca^{++}]$ this observation also seems somewhat surprising. It should be pointed out, however, that in the

region of 95—105 per cent of $[Ca_t]$ only very small inaccuracies in concentration of chelating agent would result in very marked variations in the actually induced change in concentration of $[Ca^{++}]$ This is especially marked for EGTA in this system, because of the very low conditional constant of the MgEGTA complex Thus one cannot draw definite conclusions from the experiments as to the quantitative relationship between $[Ca^{++}]$ and the increase in hydraulic conductivity

Appendix

Calculations

The calculations of the plasma $[Ca^{++}]$ and $[Mg^{++}]$ at the different plasma concentrations of EDTA and EGTA were based on the following assumptions

1) All non chelated plasma calcium and magnesium is ionized

2) At the actual concentrations of chelating agents in the plasma only calcium and magnesium will be chelated (Nicolaysen 1971)

3) The chelating agents were assumed to have the same conditional constants (= stability constans at the prevailing conditions (Ringbom 1963)) in plasma as in aquous solution at the same pH

A) At $[EDTA_t] = [Ca_t]$ in the plasma the following equations were used

1) $\frac{[CaEDTA]}{[Ca^{++}][EDTA]} = K_{CaL}$ K_{CaL} at pH 7 4 = $10^{[illegible]}$ (Ringbom 1963)

2) $\frac{[MgEDTA]}{[Mg^{++}][EDTA]} = K_{MgL}$ K_{MgL} at pH 7 4 = $10^{[illegible]}$ (Ringbom 1963)

3) $[CaEDTA]+[Ca^{++}] = [Ca_t]$ $[Ca_t]$ measured = $3 \cdot 10^{-[illegible]}$ M

4) $[MgEDTA]+[Mg^{++}] = [Mg_t]$ $[Mg_t]$ measured = $5 \cdot 10^{-[illegible]}$ M

5) $[Mg^{++}]+[Ca^{++}] = [Ca_t]+[Mg_t]-[EDTA_t]$

$[EDTA_t]$ = concentration of complexed plus free EDTA
$[Ca_t]$ = concentration of complexed plus free calcium
$[EDTA]$ = concentration of free EDTA
$[CaEDTA]$ = concentration of complexed Ca

[illegible]

The $[Mg^{++}]$ was then calculated by the use of equation 5

B) [ED[illegible]
$[EDTA_t]$ [illegible]
complexed [illegible]
all EDTA [illegible]

[illegible] as compared to $10^{[illegible]}$ [illegible] EGTA in the plasma

1) $\frac{[CaEGTA]}{[Ca^{++}][EGTA]}$ K_{CaL} $10^{[illegible]}$ (Ringbom 1963

2) $[CaEGTA]$ $[Ca_t]$ $3 \cdot 10^{-[illegible]}$ M

3) $[Ca^{++}] = [EGTA]$

[illegible] Mr. D. Sorl[illegible] for [illegible] technical assistance This work has been supported by grants [illegible] Norwegian Research Council for [illegible]diovascular Diseases The powdered [illegible] Copenhagen

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Effects of Variations in the Extracellular Osmolality on the Permeability to Nonelectrolytes of Vascular Smooth Muscle

By

OLOF JONSSON

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Abstract

JONSSON, O., *Effects of variations in the extracellular osmolality on the permeability to nonelectrolytes of vascular smooth muscle* Acta physiol scand 1971 *81* 528—539

The effects of variations in the osmolality on the general permeability properties of the smooth muscle [illegible]

smooth muscle cells are discussed

Earlier studies concerning the effects of varied extracellular osmolality on the activity of the smooth muscle cells of the rat portal vein have indicated that these effects might partly be mediated *via* alterations in the passive permeability characteristics of the cell membranes (Mellander *et al* 1967, Johansson and Jonsson 1968, Jonsson 1969 a) The purpose of the present study was to obtain information concerning the osmolality effects on the *general* permeability characteristics of the cell membranes by studying the effluxes of urea and erythritol in iso- and anisosmotic media In a concomitant article the possible *specific* effects of such tonicity changes on the permeability coefficients for sodium and potassium have been investigated (Jonsson 1971)

Urea and erythritol (mol wt 60 and 122 respectively) were considered to be suitable for the present study first because both penetrate cell membranes (Arvill, Johansson and Jonsson 1969 Johansson 1969) and, second, because they have different

oil/water partition coefficients (0 15 10^{-3} and 0 03 10^{-3}, respectively, Collander 1954, Davson 1964 p 286) This coefficient seems to be more closely correlated to the ability to penetrate cell membranes than is molecular weight *per se* (Bunch and Edwards 1969, Johansson 1970) If the transmembrane passage to some extent takes place through the hypothetical water filled pores, a change in the available pore area associated with, for instance, osmotically induced cell shrinkage should preferably alter the exchange kinetics of the molecule with the lowest oil/water partition coefficient, *i e* erythritol

Methods

The experiments were performed on portal veins from female rats of the Sprague Dawley strain with body weights varying between 200 and 250 g The animals were killed by a blow on the neck after which the abdomen was immediately opened The portal vein was carefully dissected free

normal Krebs solution (NaCl 122, KCl 4 73, $CaCl_2$ 2 49 $MgCl_2$ 1 19, $NaHCO_3$ 15 5 KH_2PO_4 1 19 glucose 11 5 mmoles/l) bubbled with a gas mixture of 96 % O_2 and 4 % CO_2 throughout the preincubation' The portal vein preparations were then used in one of the following 5 determinations 1) ^{14}C sucrose spaces 2) ^{14}C erythritol spaces 3) dry weights 4) transmembrane fluxes of urea or 5) transmembrane fluxes of erythritol The ratio between the volume and surface area of the cells was calculated from the known cell diameter assuming a cylindrical cell form Alterations in this ratio on exposures of the veins to anisosmotic solutions were estimated from the measurements of the ^{14}C sucrose spaces and the dry weights

Uptake of ^{14}C sucrose and ^{14}C erythritol

The experimental procedures applied to determine the uptake of radioactive tracer substances in the rat portal vein have been described in detail elsewhere (Arvill *et al* 1969) In the experiments where the uptake of ^{14}C erythritol were to be measured the veins were transferred to flasks containing 2 ml normal Krebs solution to which ^{14}C erythritol with a specific activity of 2 3 mc mmole was added to a concentration of 0 90 mmole/l After 15 30 60 or 120 min in this solution at 37 C the muscles were quickly blotted between 2 pieces of filterpaper weighed on a Cahn electrobalance and homogenized in 10 % tri-chloro-acetic acid (TCA) The radioactivity of the muscle extracts and the media were determined with a Packard Tri Carb liquid scintillation counter (for details see Arvill *et al* 1969)

The uptake of ^{14}C sucrose was measured at 15 C Therefore after the preincubation period the muscles were allowed to adapt to this low temperature for 40 min in normal Krebs solution or when

centration of 0 25 mmole l After 15 min in these media the incubations were interrupted and the muscles treated exactly as those exposed to ^{14}C erythritol All media used were bubbled for one hour with a gas mixture of 96 % O_2 and 4 % CO_2 previous to the experiments

Dry weights The time schedule and incubation solutions were the same in the experiments where the dry weights of the portal veins were measured as in those described above where the uptake of ^{14}C sucrose was determined After blotting and weighing (wet weight the portal veins were dried over night in a vacuum oven and then reweighed dry weight) The dry weights are expressed in per cent of the corresponding wet weights

Measurements of urea and erythritol effluxes

ture Furthermore when the composition of the washout solution was different from that of normal Krebs the last 15 min of ^{14}C urea or ^{14}C erythritol exposure was performed in a medium identical

with the actual test solution These arrangements were made in order to achieve steady state condi-

These media, as well as those used for the loading of the veins, had been bubbled with a gas mixture of 96 % O_2 and 4 % CO_2 before the experiments for at least one hour The amount of radioactivity in the tissue at the end of the efflux was determined as described above for the uptake experiments and added to the activity in the successive samples in reverse order, giving the amount of radioactivity in the portal veins as a function of time The remaining activity at the different time intervals was recalculated into per cent of the initial activity to facilitate the comparison of different washout curves It should be noticed that the composition of the medium was never altered during one and the same washout The degree of quenching turned out to be the same for extracts, incubation media and washout solutions in these experiments as well as for the extracts and incubation media of the uptake experiments described above

The analysis of the washout curves were performed with the aid of a calculator (Hewlett-Packard 9100 A) Correlation and regression coefficients as well as the intercepts with the ordinate were calculated for the best fitting line in a semilogarithmic plot The test began with the last two points whereafter the earlier ones were taken into account one by one in reverse order The washout was

an objective and standardized analysis of the washout experiments

Measurements of the osmolality

Results

Urea fluxes Preliminary experiments performed at 37° C showed that the washout of ^{14}C urea at this temperature was very fast In order to slow down the rate of exchange the remaining washout experiments with this tracer were performed at 15° C Fig 1 illustrates a representative ^{14}C urea washout curve obtained in normal Krebs solution after 90 min of exposure to the active solution at 37° C followed by another 30 min at 15 C, a loading period that is quite sufficient for the tracer to equilibrate in the total water (Arvill *et al* 1969) The decrease in the remaining activity of the vein expressed in per cent of the initial activity (triangles), is plotted versus time After the first seven minutes the reduction of the radioactivity left in the tissue could be fitted by a single exponential with a rate constant of 0 050 min $^{-1}$ When this slower component of the washout curve was extrapolated to zero and subtracted from the total curve a faster component (closed circles) with a rate constant of 0 95 min $^{-1}$ was obtained The intercept with the ordinate of the slower component had a value of 23 9 per cent which conformed to a volume of 26 7 ml 100 g wet weight The corresponding values for the fast components were 38 2 per cent and 42 8 ml 100 g wet weight, respectively The other 37 9 per cent of the ^{14}C urea were washed out so rapidly that a rate constant for it could not be determined

The mean values ± SE for seven similar washout curves were for the slower component 0 0527 ± 0 0017 min $^{-1}$ and 23 3 ± 0 8 ml 100 g wet weight and for the faster component 0.85 ± 0 05 min $^{-1}$ and 36 0 ± 2 7 ml 100 g wet weight, respectively The

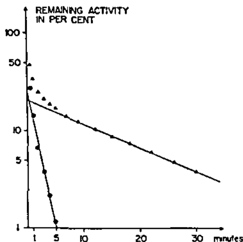


Fig 1

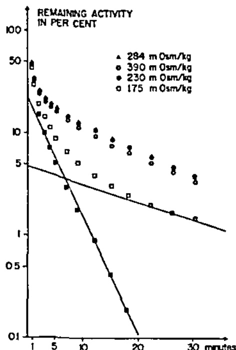


Fig 2

Fig 1 Efflux of [14]C urea from rat portal vein at 15° C The triangles represent the logarithmic decrease of activity as a function of time The monoexponential part of this curve is extrapolated to zero time and subtracted from the total remaining activity, giving the values for the faster component (filled circles)

Fig 2 Efflux of [14]C urea at 15° C at four different extracellular osmolalities The late monoexponential part of the curve obtained at 175 mOsm/kg is extrapolated to zero time and subtracted from the total remaining activity giving the values for the faster component (filled squares)

values given below for total water and extracellular space during the present conditions indicate that the volume of cell water is approximately 270 ml/kg wet weight It therefore seems likely that the slow component represented exchange of intracellular urea The extracellular space measured by [14]C sucrose uptake experiments was considerably greater than the intercept with the ordinate of the faster component (52 8 ml/100 g wet weight compared to 36 0 ml/100 g wet weight) This is, however, not incompatible with the conclusion that this phase represented washout of extracellular urea since it might well be that the tracer substance in the most "superficial parts" of the extracellular space has a still higher exchange rate Such an interpretation is supported by the finding that the earliest points obtained by subtraction of the slowest component from the total washout curve deviated from the faster component

The fast and slow components can be described by the exponential terms $A\,e^{-\lambda_1 t}$ and $B\,e^{-\lambda_2 t}$, where A and B are the initial amounts of [14]C urea in the two phases and λ_1, λ_2 are the rate constants and t the time in min after the transfer from the active solution However, due to the fact that the cellular phase is coupled in series with the

extracellular phase, there will occur an interaction between the two exponentials leading to an overestimation of the intracellular amounts of urea. The true intercept (B') with the ordinate of the slow component can be obtained from the formula

$$B' = \frac{AB(\lambda_1-\lambda_2)^2}{A\lambda_1^2+B\lambda_2^2} \quad (1)$$

(Huxley 1960).

The corrected value for B thus obtained corresponded to a volume of 20.5±0.7 ml/100 g wet weight. This figure estimates the volume of the intracellular fluid, *provided ^{14}C urea has the same concentration in this fluid* as in the incubation medium at the end of the 120 min long loading period.

The influence of the interaction between the two exponentials on the value of λ_2 (*i.e.* rate constant of the cellular fraction) could in the present case be neglected since it was deduced from that part of the washout curve where it had straightened out to a single exponential. Representative washout curves of ^{14}C urea obtained at four different extracellular osmolalities are illustrated in Fig. 2. As mentioned in Methods the osmolality was increased by adding sucrose and decreased by removing sodium chloride. The triangles (almost completely covered by the closed circles) represent washout in normal Krebs solution, the open circles Krebs plus 100 mmoles sucrose/l, the filled circles Krebs minus 30 mmoles NaCl/l and the open squares Krebs minus 60 mmoles NaCl/l. The curves obtained at the more moderately changed osmolalities were almost identical contrary to the one obtained at 175 mOsm/kg which deviated markedly from the others in that it exhibited a steeper decline and did not straighten out to a clearcut single exponential. Only the last three points could be fitted by a single exponential with a correlation coefficient less than −0.996. However, in control experiments it could be demonstrated that this late monoexponential phase represented the decrease in remaining activity for at least another 18 min. If this slow component was subtracted from the washout curve, a faster intermediate exponential was obtained (filled squares) which in all similar experiments consistently prevailed in the interval 4 to 18 min. The intercept of the slowest component with the ordinate corresponded to a volume of 6.0±0.9 ml/100 g wet weight and of the intermediate component to a volume of 25.7±2.0 ml/100 g wet weight (means±SE from 5 washout curves, uncorrected values). A possible interpretation of these two phases is that the smaller component represents washout of urea contained in the nucleus or some other cellular subcompartment, and the intermediate component urea contained in the cytoplasm. This conclusion is supported by the finding that the volume of the nucleus of smooth muscle cells is comparatively large (Goodford and Hermansen 1961). If the abovementioned interpretation is correct, the transmembrane passage of urea was represented by the intermediate component (rate constant 0.254±0.010 min^{-1} (mean±*SE of 5 washout curves*)).

The mean values±SE for rate constants and intercepts with the ordinate (corrected and recalculated to urea spaces) for the cellular component of the washout curves performed at 390 mOsm/kg were 0.0582±0.0020 min^{-1} and 20.4±0.9 ml/100

g wet weight (n = 7), respectively and for those performed at 230 mOsm/kg 0 0527 ± 0 0020 min⁻¹ and 18 4 ± 1 4 ml/100 g wet weight (n = 6), respectively

Erythritol fluxes The exchange of erythritol was comparatively slow and therefore these experiments could be performed at 37° C Three representative washout curves obtained at 284, 444 and 175 mOsm/kg are illustrated in Fig 3 The muscles had been exposed for 60 min to active solution prior to the washout It was learned from uptake experiments that 60 min of incubation was quite sufficient for ^{14}C erythritol to reach a plateau value of 82 8 ± 1 5 ml/100 g wet weight (n = 3) which fairly well agreed with the total water content of the veins under these conditions (79 3 ml/100 g wet weight, Jonsson 1969 b) The loss of ^{14}C erythritol could in all three cases be described by a single exponential after 8 min The mean values (±SE) for rate constants (min^{-1}) and intercepts with the ordinate (corrected and recalculated to erythritol spaces, ml/100 g wet weight) for the monoexponential parts of the curves were for those obtained with normal Krebs (284 mOsm/kg) 0 0222 ± 0 0006 and 18 1 ± 0 6 (n = 11), with Krebs plus 150 mmoles sucrose/l (444 mOsm/kg) 0 0290 ± 0 0010 and 18 1 ± 0 9 (n = 8), with Krebs minus 60 mmoles NaCl/l (175 mOsm/kg) 0 0290 ± 0 0006 and 16 6 ± 0 4 (n = 7), respectively

Though the intercept of the slow component corresponded to a space somewhat smaller than the amounts of intracellular water (267 ml/kg wet weight), it seems reasonable to assume that this component represented transmembrane exchange of erythritol

Calculation of permeability coefficients Permeability coefficients in cm/sec can be calculated for uncharged molecules with the formula

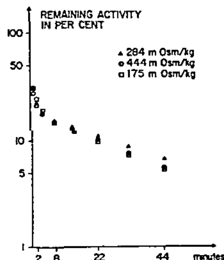


Fig 3 Efflux of ^{14}C erythritol at 37 C at three different extracellular osmolalities

$$P = \frac{V}{A} \cdot \lambda \qquad (2)$$

which is derived from Fick's law of diffusion under the assumptions that the concentration of the tracer substance in the washout medium can be neglected and that the $\frac{V}{A}$ ratio is constant throughout the washout (Davson 1964 p 280) $\frac{V}{A}$ stands for the volume surface ratio and λ for the rate constant The frequent exchange of the washout flasks in the present study prevented the tracer concentration in the washout medium from becoming more than a very small fraction of that in the cell water which justifies the assumption presented above

The diameter of the smooth muscle cells of rat portal veins, stretched to their *in situ* length, is approximately 4 μ as measured from sections of rapidly frozen preparations (Haglund, Jodal and Jonsson, unpublished observation) This figure, which agrees well with the measured cell diameter of other types of vascular smooth muscle preparations (Rhodin 1967), gives a $\frac{V}{A}$ ratio of 10^{-4} cm assuming a cylindrical cell form It has previously been reported that the smooth muscle cells behave as perfect osmometers on exposure to hyperosmotic solutions (Arvill *et al* 1969) If the reduction in cell volume is considered to be accomplished solely by a reduced cell diameter, the $\frac{V}{A}$ ratio is lowered from 10^{-4} cm to 0 85 10^{-4} cm in Krebs plus 100 mmoles sucrose/l and to 0 80 10^{-4} cm in Krebs plus 150 mmoles sucrose/l

It is somewhat more complex to calculate the $\frac{V}{A}$ ratio for muscles incubated in hypo-osmotic media since it has been shown that the cells of the rat portal veins only under certain conditions behave as perfect osmometers on reductions in the osmolality (Jonsson 1969 b) In this article it was shown that the volume of the cell water increases only by 14 per cent when veins, stretched to their *in situ* length, are incuba

TABLE 1

Incubation medium	Sucrose-^{14}C space ml/100 g Mean SE	n	P_{max}	Dry weight per cent of wet weight Mean SE	n	P_{max}	Intracellular water per cent Mean SE	P_{max}
Normal Krebs	52 8 0 9	13	0 1	20 5 0 3	9	0 7	100	0 25
Krebs—30 mmoles NaCl/l	50 5 0 8	8	0 2	20 2 0 4	9	0 001	113 7	0 02
Krebs—60 mmoles NaCl/l	50 2 – 1 0	5		18 8 0 3	9		126 8	

ted for 15 min in Krebs minus 60 mmoles NaCl/l at 37° C. This corresponds to a $\frac{V}{A}$ ratio of $1.07 \cdot 10^{-4}$ cm, a figure that can be used for calculating the permeability coefficient to erythritol for which the washout experiments were performed at 37° C.

Experiments were carried out in the present study to determine the changes in $\frac{V}{A}$ ratio caused by graded osmolality reductions at 15° C, the temperature at which the ^{14}C urea washout experiments were performed. The method, applied for determining the volume of the cell water under different experimental conditions as well as the concomitant calculations, have been described in detail in earlier articles (Arvill *et al.* 1969, Jonsson 1969 b). The extracellular space has been considered to be best measured by the ^{14}C sucrose space after 15 min of equilibration. Such "15 min ^{14}C sucrose spaces" together with corresponding dry weights are given in Table I for muscles, stretched to their *in situ* length, incubated in either Krebs minus 30 mmoles NaCl/l or Krebs minus 60 mmoles NaCl/l at 15° C. The changes in the weights of the intracellular water caused by hypo-osmolality have then been calculated according to Jonsson 1969 b. The values thus obtained are given in Table I. The weight of cell water of veins incubated in normal Krebs solution has been arbitrarily set at 100 g. The $\frac{V}{A}$ ratios calculated from these figures, assuming the cell diameter to be 4 μ in normal Krebs solution, are $1.06 \cdot 10^{-4}$ cm and $1.12 \cdot 10^{-4}$ cm for the veins

Table II

Tracer substance	Washout medium	Temperature	Rate constant min⁻¹ Mean ± SE	n	P_{max}	Permeability coefficient cm/sec
^{14}C urea	Normal Krebs 284 mOsm/kg	15° C	0.0527 ± 0.0017	7	0.1	$8.78 \cdot 10^{-8}$
^{14}C urea	Krebs + 100 mmoles sucrose/l 390 mOsm/kg	15° C	0.0582 ± 0.0020	7	1.0	$8.27 \cdot 10^{-8}$
^{14}C urea	Krebs—30 mmoles NaCl/l 230 mOsm/kg	15° C	0.0527 ± 0.0020	6	0.001	$9.31 \cdot 10^{-8}$
^{14}C urea	Krebs—60 mmoles NaCl/l 175 mOsm/kg	15° C	0.254 ± 0.010	5		$4.74 \cdot 10^{-7}$
^{14}C erythritol	Normal Krebs 284 mOsm/kg	37° C	0.0222 ± 0.0006	11	0.001	$3.70 \cdot 10^{-8}$
^{14}C erythritol	Krebs + 150 mmoles sucrose/l 444 mOsm/kg	37° C	0.0290 ± 0.0010	8	0.001	$3.87 \cdot 10^{-8}$
^{14}C erythritol	Krebs—60 mmoles NaCl/l 175 mOsm/kg	37° C	0.0290 ± 0.0006	7		$5.41 \cdot 10^{-8}$

incubated in Krebs minus 30 mmoles NaCl/l and Krebs minus 60 mmoles NaCl/l respectively

The urea and erythritol permeability coefficients, calculated by equation (2) using the data given above for portal veins incubated at different extracellular osmolalities, are presented in Table II together with the corresponding rate constants The increase in rate constant observed for both urea and erythritol in hypertonic solutions can almost completely be ascribed to the reduced $\frac{V}{A}$ ratio without postulating any alteration in the permeability coefficient It seems, however, safe to conclude that such an alteration occurs on exposure to Krebs minus 60 mmoles NaCl/l since the increase in rate constant and in $\frac{V}{A}$ ratio can then be said to co operate The slight change in permeability coefficient observed in Krebs minus 30 mmoles NaCl/l cannot be considered statistically significant

Discussion

The concept of a barrier function of the vascular smooth muscle cell membranes that limits the rate of exchange of solutes between the muscle cells and the surrounding medium has been adopted in the interpretation of the results obtained in the present as well as in the concomitant study (Jonsson 1971) The reason for this concept concerning the question whether the properties of the cell membranes or those of the cytoplasm (for ref see Jones 1970) regulates the fluxes of solutes is that the effluxes of urea and erythritol could after a while be represented by a clear cut single exponential whose extrapolated intercept with the ordinate corresponded to a volume that agreed satisfactorily with the volume of the cell water calculated from dry weights and ^{14}C sucrose spaces Furthermore in the concomitant study (Jonsson 1971) evidence has been put forward that the exchange of potassium and sodium in the portal vein can be represented by a simple exponential term i e that the cell membranes at least to some extent restrict the effluxes of solutes

The values for the urea and erythritol permeability coefficients presented above have been calculated by the use of the Fick equation One assumption in doing this was that the rate of penetration through the cell membranes is small compared to the rate of diffusion within the cells and in the bathing solution Only if this is true it is justified to replace ΔC by

$$\frac{C_{in} \quad C_{out}}{\text{membrane thickness}}$$

where C_{in} and C_{out} denote the concentrations in the internal and external solutions respectively However, if for some reason a concentration gradient would occur on any side of the membrane the permeability coefficients calculated in this way would be smaller than the true ones

Another point that should be stressed concerning the calculation of the P values at the different extracellular osmolalities is that the estimated changes in the cell volume/cell surface ratios are probably somewhat overestimated Such a possible bias could be due to the fact that a percentual alteration in the weight of the cell water has been taken to represent the corresponding change in the total cell volume, though without doubt some portion of the dry weight material must be considered to be intracellular Further, it could be due to the fact that the change in cell volume solely has been ascribed to an alteration in the cell diameter Since the magnitude of the adjustments necessary to correct these two possible partialities are not known, it was considered most appropriate to use the above computed 'maximal alterations" in the V/A ratios These uncertainties do not, however, influence the conclusion that the P values for both urea and erythritol are decidedly increased in the drastically hypo-osmotic medium (Krebs minus 60 mmoles NaCl/l) since the changes in the V/A ratio and in the rate constants co-operate in this case and the variation in the latter parameter is *per se* statistically significant

As mentioned above the intercept with the ordinate of that part of the washout curve which was taken to represent transmembrane exchange gave values for the cell water in ml/100 g wet weight that fairly well agreed with that calculated from the ^{14}C sucrose space and dry weight It might seem confusing that the value for this intercept was changed so little on osmolality alterations This apparent contradiction may be explained by the fact that the wet weight of the veins is changed simultaneously with the cell volume in such a way that the variation in the ratio cell volume/wet weight of the preparation is considerably hampered (Johansson and Jonsson 1968, Jonsson 1969 b)

The present two studies concerning the effects of altered extracellular osmolality on the permeability properties of the vascular smooth muscle cell membranes were initiated by the observation that the pronounced effects on the spontaneous activity of the portal veins, caused by osmotically induced variations in the cell volume, could not be fully ascribed to the concomitant changes in the transmembrane ionic gradients (Mellander *et al* 1967, Johansson and Jonsson 1968 Jonsson 1969 a) It was therefore suggested that alterations in cell volume, beside their influence on the concentrations of the intracellular ions, may also affect the permeability characteristics of the cell membranes in such a way that the ratio between the permeability coefficients to sodium and potassium vary inversely to the extracellular osmolality (Johansson and Jonsson 1968, Jonsson 1969 a) This hypothesis has been examined in the following article (Jonsson 1971) while the purpose of the present study was to obtain some information concerning the general permeability qualities at different tonicities

The ability of different nonelectrocytes to penetrate the cell membranes of the portal vein has been demonstrated to be mainly correlated to the lipid solubility of the substances, but also to their molecular weights (Johansson 1969, 1970) The influence of the molecular size was considered in terms of a porous membrane model in which the hydrophilic pores controlled the rate of penetration of substances with low lipid solubility It should, however, be stressed that these polar routes turned out to play a

comparatively little role in the portal vein compared to, for instance, in the rat erythrocytes (Johansson 1970) Nevertheless it is conceivable that a varied degree of stretch of the cell membranes, caused by an osmotically induced change in the cell volume, may alter the permeability properties, either by influencing the thickness of the lipoid membrane structures or by affecting the mean area of the "pores"

The results presented above do not indicate any alteration in the general permeability properties on exposure to hyperosmolality or on moderate reductions in the tonicity However, upon more drastic decreases in osmolality there is a clearcut increase in the P values for both urea and erythritol The finding that the permeability coefficient for urea is considerably more increased than that for erythritol on drastic reductions in osmolality do not seem to conform with the hypothesis that the increases in permeability observed are due to widening of waterfilled pores The reasons are first, that urea has the highest lipoid solubility of the two substances, and second, that the molecular size of urea is considerably smaller than that for erythritol (2 03 and 3 06 Å respectively, Goldstein and Solomon 1960) According to the connections between the size of molecules and their ability to pass cell membranes (Davson 1964 p 294) for different transverse section areas of the polar routes the relative decrease in Staverman reflexion coefficient as a result of pore widening should, in point of principle, be most pronounced for the largest of the penetrating molecules It therefore seems more plausible that the reduction in the rate limiting properties of the cell membranes, observed on exposure of the portal vein preparations to Krebs minus 60 mmoles NaCl/l is due to some other mechanism, such as some interference with the lipoid membrane structures

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Unit Responses in the Rat Cochlear Nucleus to Tones of Rapidly Varying Frequency and Amplitude

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Abstract

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The response of single units in the cochlear nucleus of the rat to sweep tones (FM sounds) and short tone bursts (AM sounds) has been investigated. It was found that the response pattern to FM sounds became more restricted with regard to tone frequency at a certain rate of tone frequency change. The probability of firing within a narrow frequency range surrounding the unit's CF became frequently more than ten times greater than that at low sweep speeds.

For a certain range of higher sweep rate, cycle histograms become narrower and higher than at low sweep rates. The range of frequency change where the peaks had maximal height was found to lie in the region between 1 and 15 MHz/sec for units with CF from 5 to 30 kHz. This effect was found to persist over a sound intensity range of more than 60 dB but was less marked at very low intensity levels. The threshold of the units investigated did not become lowered at any rate of frequency change.

The cycle histograms of the response to sounds of constant frequency and variable duration (AM sounds) differed significantly from that to FM sounds. Only occasionally was an increase in height of the cycle histograms seen when the duration of the sounds was narrowed and the repetition rate increased.

The response areas of single units at different levels in the auditory system have been studied in numerous previous investigations using pure tones with a constant or slowly varying frequency. In many studies a unit's response area has been expressed by its threshold as a function of tone frequency (tuning curves, cf. Kiang 1965), but it has been claimed that the responses above threshold offer a more representative description of the characteristics of a neuron than threshold values. Iso-rate functions which show the sound intensity required to evoke a certain increase in firing rate represent one way of describing a unit's response area above threshold. Such curves however are essentially parallel to the turning curves (cf. Møller 1969 a). Other authors have plotted the response area as a function of tone frequency, with the sound intensity as parameter (see e.g. Hind et al. 1967).

Although most natural sounds have a more or less rapidly changing spectral distribution, only a few neurophysiological investigations on the response to fre-

quency modulated sounds have been reported in the literature Suga (1965) studied the threshold of single units in the cochlear nucleus and inferior colliculus to FM sounds similar to the echo-locating sounds of flying bats In his detailed studies of units in the cochlear nucleus he found no appreciable difference between the threshold of FM sounds and that of tones of constant frequency Watanabe and Ohgushi (1968) examined the threshold to FM sounds in different parts of the ascending auditory pathways Though they found no specificity of the neurons in the cochlear nucleus with regard to direction of FM sweep at higher levels in the auditory pathway they found a rather pronounced directionality in a number of units These features were especially marked in the auditory cortex (AI), which agreed with earlier findings by Whitfield and Evans (1965) Erulkar *et al* (1968) investigated the response of cochlear nucleus units to stimulation with trapezoidally FM modulated sounds above threshold They found only slight differences between the responses to slow and to fast sweep-rates in units in the cochlear nucleus

In a previous investigation (Møller 1969 b), similar sounds were used The range of the frequency sweep however, extended over a much wider frequency range than it did in the above mentioned studies It was found that the response areas of single neurons in the cochlear nucleus to tones of constant or slowly varying frequency (the static response areas) differed significantly from those obtained with tones whose frequencies varied rapidly (the dynamic response areas) The intensity of the sounds was 20—30 dB above threshold At low sweep rates, the distribution of nerve impulses as a function of tone frequency was in accordance with the unit s response area to tones of constant frequency At higher sweep-rates however more discharges were evoked near the unit s characteristic frequency (CF) than was the case at lower sweep-rates This was illustrated in the cycle histograms of the responses to sounds with triangulary modulated frequencies In these histograms a peak occurred at a tone frequency which was equal to the unit s CF The height of the peak reached a distinct maximum at a certain sweep rate Above this sweep-rate the peak again became wider and lower The mean firing rate changed only slightly with sweep rate and in several units not at all (Møller 1969 b)

This paper is concerned with the coding of sweep-tones in single units in the cochlear nucleus of the rat and is an extension of the above mentioned study (Møller 1969 b) The present paper describes in greater detail the relationship between rate of frequency change and the distribution of nerve impulses in a wide range of sound intensity To assess whether or not it is the transient character of the response to FM sounds that is responsible for the most localized response to fast FM sweeps the response pattern to FM sounds is compared with the response to sounds of constant frequency and variable duration

Methods

White rats weighing from 250—350 g were used in this investigation The rats were anesthetized by intraperitoneal injection of urethane (1 5 g/kg b w) Temperature was maintained near 37° C in the normal way The trachea was cannulated and a hole was made in the

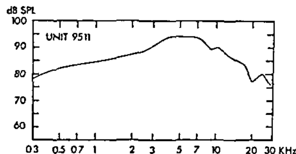


Fig 1 A Frequency response of the sound system Sound pressure level (in dB re 0 0002 μb), measured close to the eardrum of a rat, is shown as a function of tone frequency The graph shows the sound pressure for a 1 volt RMS input to the earphone in a typical experiment

occipital part of the skull The outer ear was removed and the head was mounted in a head holder (described earlier, Møller 1969 a) Part of the cerebellum was sucked away to make the cochlear nucleus visible, and the animal was then placed in a sound proof box Recordings ... M KCl ... ode was ... sound ... rded on ... chnique ... phase of the modulation wave This served the purpose of guiding the experimenter in choosing suitable ...

4134) The frequency characteristic of the complete sound generating system is shown in Fig 1 A The frequency modulated tones were generated by a Wavetek function generator (type 112) which was frequency modulated with another Wavetek generator (type 110) The frequency modulated tones used in this investigation were similar to those used in a previous investigation (Møller 1969 b) but in the majority of the experiments reported on in the present investigation trapezoidal modulation was used instead of triangular modulation

Triangular frequency modulation implies that the tone frequency changes continuously during the entire modulation cycle, while the frequency of a trapezoidally modulated tone changes

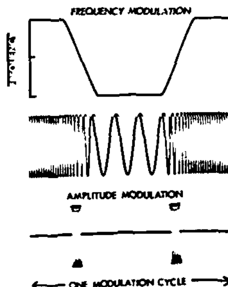


Fig 1 B The wave form of the trapezoidally frequency modulated sound (above) and the amplitude modulated sound For both types of modulation one complete cycle of the modulation is shown

only during a certain fraction of the modulation cycle but otherwise is steady When using triangular modulation of constant extent of frequency deviation the repetition rate and the rate of changes in tone frequency are linked together Trapezoidal modulation however makes it possible to vary the rate of tone frequency change independently of the rate of the modulation

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produces a mean excitation within a certain frequency band independent of the sweep-rate When the sweep frequency is doubled for example the tone passes the unit s response area twice as fast but its duration is only half as long For trapezoidal modulation various wave shapes were used in order to alter the fraction of the modulation cycle where the tone frequency varied

Sounds of variable duration and constant frequency (AM sounds) were produced by gating the Wavetek type 112 generator with triangular waves from the Wavetek type 110 generator The duty cycle (ratio between tone and silence) could be varied by adjusting the trigger level control on the Wavetek 112 Usually a duty cycle of 1 10 was used The frequency and amplitude modulated signals are illustrated in Fig 1 B In additional investigations continuous *tones of constant frequency generated with a Hewlett Packard tone generator were super* imposed on the FM tones The sound system had two channels with independent attenuators

The DIDAC 800 was modified in such a way that its address register could be reset by the trigger pulse immediately before it initiated the sweep This made it easier to make cycle histograms since each trigger pulse automatically reset the sweep to its initial position before initiating a new sweep This left the experimenter free to use a fraction of the channels of the analyzer in order to obtain optimal time resolution in the analysis

Integrated histograms were also produced From these the total number of discharges as well as the number of discharges contained in each peak of the histogram were determined

Results

The results of the present study are based on analysis of spike data from 55 units in 15 animals *Only units from which satisfactory recordings were made for periods* of time longer than 30 min were included Recordings from many of the units were made over periods of 2 hrs or longer

When the frequency of a tone varies slowly an auditory unit s discharge frequency varies as a function of tone frequency in a manner similar to the response area of the unit measured with steady tones The response evoked by a tone whose frequency was equal to that of the unit s CF could be suppressed in most units by a second tone of a slightly higher frequency In some units a tone whose frequency was lower than the unit s CF had a similar but usually less pronounced effect In many of the units which had spontaneous activity a similar depression of the spontaneous activity was seen for tones in a limited range above the unit s CF This depression has previously been termed inhibition but the results of *recent investigations (see e g* Hind 1970) suggest that it may be a form of modulation rather than a neural inhibition in the sense in which it is usually defined

Fig 2 shows typical responses of a unit in the cochlear nucleus to trapezoidally frequency modulated tones The figure shows histograms of the response to one min of stimulation at two different sweep rates a slow rate (0 1 sweep/sec) and a fast sweep rate (6 4 sweeps/sec) which latter is near that of maximal sharpening The corresponding rates of change in tone frequency are 7 3 and 470 kHz/sec respectively It can be seen that the peaks in the histograms are much higher at a

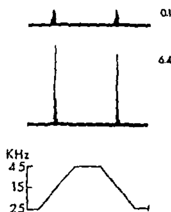


Fig 2 Cycle histograms of the responses to trapezoidally frequency modulated tones with a rate of 0 1 and 6 4 sweeps/sec corresponding to a rate of change in tone frequency of 7 3 and 470 kHz/sec respectively In the lower part of the figure the shape of the frequency modulation is illustrated

sweep rate of 6 4 (lower histogram) than at 0 1 sweeps/sec (upper histogram) In addition the peaks in the lower histogram are much narrower than those in the upper The unit s CF was 15 0 kHz The intensity of the tone at the CF was 50 dB SPL or 15 dB above the threshold of the unit The tone frequency of the stimulation varied symmetrically around the unit s CF

Fig 3 shows the variation in the height of the peaks in the histograms of the responses of the unit shown in Fig 2 to trapezoidally frequency modulated tones in a large range of sweep-rates The tone frequency varied from 4 5 kHz to 25 kHz The change in tone frequency occupied 60 % of the modulation cycle Fig 3 A B and C represent the responses for sound intensities of 25 15 and 5 dB above threshold at CF respectively The responses to rising tone frequency are represented by solid lines and those to falling tone frequency by dashed lines It can be seen that the peaks in the histograms of the responses to FM tones increase in height when the sweep rate is successively increased from low sweep rate up to a certain optimal sweep-rate Above this optimal rate on the other hand the height of the peaks in the histograms decreases rapidly The figure also shows that for higher sound intensities the maximum peak height is reached at a higher sweep frequency At a sound level of 25 dB above the unit s threshold the height of the peaks in the histograms reaches a maximum at a sweep frequency of about 12 Hz which corresponds to a rate of change in tone frequency of about 0 9 MHz/sec At lower intensities the maximum in the histograms occurs at a somewhat lower sweep rate but the shape of the curves is almost independent of sound intensity in the investigated range (A B and C in Fig 3) It can also be seen from these curves that the direction of the sweep has little influence on the response except at very high sweep-rates

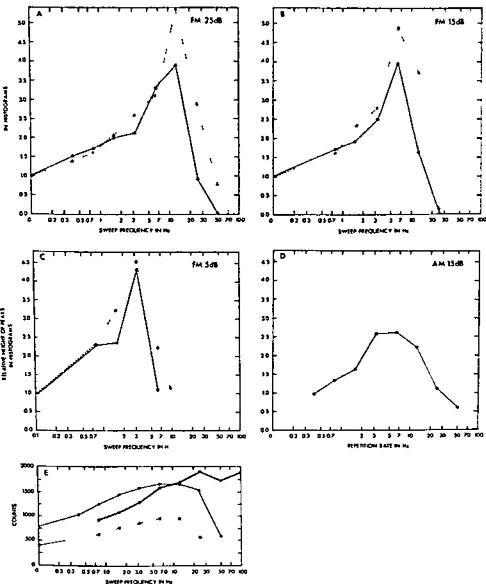


Fig 3 Responses of a unit whose CF was 15 0 kHz The unit showed no spontaneous activity *A—C* Relative height of the peaks in the cycle histograms of the response to trapezoidally frequency modulated tones shown as a function of sweep rate in Hz The tone frequency changed during 60 % of the modulation cycle The graphs were obtained at three different sound intensities, as indicated by legend numbers (in dB re threshold at CF) Continuous lines represent rising frequency sweep and dashed lines falling frequency sweep

D Relative height of the peaks in the cycle histograms of the response to repetitive tone bursts The ratio between sound and silence was 1 10 throughout the range of repetition rates shown in the graph The sound intensity of each burst was 15 dB above the unit s threshold at CF

The total number of spikes evoked during a one minute recording period is shown in Fig. 3 E as a function of sweep frequency. The thin continuous line represents the response at 25 dB and the dashed line the response at 15 dB above threshold. Both curves show a broad maximum located in the range of the same sweep frequency as that of the maximum in the height of the peaks in the histograms but this maximum is much less pronounced.

The height of the peaks in the histograms of the responses to short tone bursts (*AM sounds*) is shown in Fig. 3 D as a function of repetition rate. The tone frequency of the bursts was equal to the unit's CF and the intensity was 15 dB above its threshold. The curve has a maximum around a repetition rate of 3—7 Hz *but in this case the maximum is much broader* and not as high as *for the* curves showing the response to FM sounds (Fig. 3 A to C). The ratio between tone and silence was kept constant at 1:10 in the range of repetition rates used. The duration of the tone thus varies in accordance *with the repetition rate*.

The unit whose responses are shown in Fig. 2 and 3 had a tuning curve of the *normal type with a very steep high frequency skirt (cf. Moller 1969 a)* and it had no spontaneous activity. When a 15 kHz continuous tone (the unit's CF) was sounded together with a tone of slowly varying frequency, the histograms of the unit's response showed a typical area of suppression above the unit's CF.

The response of another unit which behaved in a slightly different way is shown in Fig. 4. This unit had a CF of 22.0 kHz and a moderate degree of spontaneous activity. The response of this unit to trapezoidally frequency modulated tones was studied in the intensity range from 25 to 65 dB above its threshold at CF (45 to 85 *dB SPL at 22 kHz). The tone varied from 4.5 kHz to 30 kHz* and the variation occupied 15 % of the modulation cycle. The relative height of the peaks in the cycle histograms of the response to FM sounds is shown in Fig. 4 A, B and E. The *corresponding total spike* counts during one minute of stimulation are shown with heavy lines in Fig. 4 C, D and G. In these graphs the number of nerve impulses contained in each of the two peaks in the histograms is also shown, the thin continuous lines representing the responses for rising tone frequency and the dashed lines those for falling tone frequency.

In this unit there is a marked difference between the response to rising and falling tone frequency, especially at the highest intensity used. In particular the maximum height of the peaks in the cycle *histograms in Fig. 4 A, B and F reveals a greater* dependency on the intensity and this is much more pronounced for rising frequency sweep than for falling sweep. At the lowest intensity used (25 dB above threshold) the maximum in the *height of the peaks in the histograms occurs around* 12 Hz (Fig. 4 F) corresponding to a rate of change in tone frequency of 4 MHz/sec. At this sound intensity increasing and decreasing frequency sweeps yield similar

F. Total numbers of spike counts during 1 min of stimulation. The thin line represent FM sounds, continuous line of 25 dB and dashed line of 15 dB above threshold at the unit's CF. Heavy line represents the response to AM sounds with a sound level of 15 dB above the unit's threshold.

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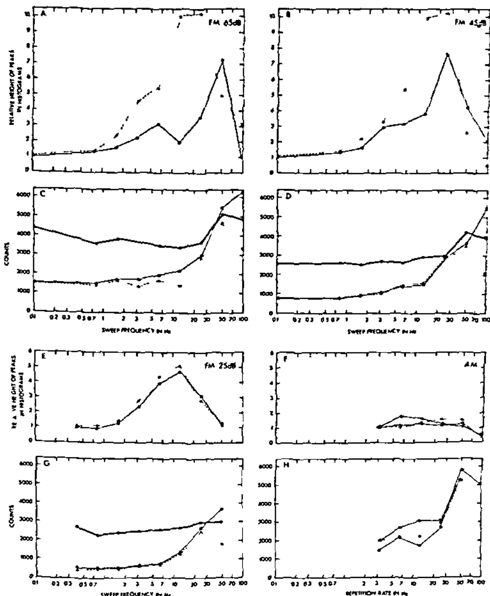


Fig 4 Responses of a unit whose CF was 22 0 kHz The unit showed a moderate degree of spontaneous activity

dashed lines falling frequency sweeps

C, D and G The corresponding total number of spikes during 1 min of stimulation (heavy lines) and the number of spikes contained in each peak for rising (thin continuous lines)

responses At an intensity of 45 dB above threshold, the histogram peaks corresponding to decreasing frequency sweep have a broad maximum between 25 and 50 Hz The maximum for the increasing tone-frequency sweeps has moved up to 20 Hz and is smaller than for the decreasing tone-frequency sweeps (Fig 4 B) When the intensity is further increased to 65 dB above threshold, the maximum for increasing frequency sweep has moved further up in frequency, to about 50 Hz, while the location of the broad maximum of the relative height of the peaks in the histograms of the responses to decreasing tone-frequency sweep has remained almost unchanged

The total number of discharges varies only slightly as a function of sweep frequency (heavy line in Fig 4 C, D and G) The number of discharges within each peak in the histograms shows, however, a marked increase in the sweep-range from 15 to 100 Hz (Fig 4 C D and G, continuous and dashed thin line) This implies that a large fraction, or almost all nerve impulses are located within the two peaks in the histograms at high sweep frequency From Fig 4 A, B and E, however, it follows that the sweep frequency where the total number of nerve impulses reaches its maximum is significantly higher than the frequency where the heights of the peaks in the histograms are maximum

In Fig 4 F, the relative height of the histograms of the response to AM sounds is shown (The AM sounds were of the same type as used in the experiment depicted in Fig 3) The curves represent the responses to different sound-intensities of the AM-sounds Open circles represent 65 dB triangles 45 dB and filled circles 25 dB above the unit s threshold

From Fig 4 F, it can be seen that the unit s response pattern to repetitive tone-bursts of constant frequency is almost independent of the repetition rate and thus of the duration of the tone-bursts Moreover the response pattern changes remarkably little when the intensity is changed from 25 to 65 dB above threshold The total number of nerve impulses contained in the peaks in the cycle histograms during 1 min of stimulation can be seen in Fig 4 H The curves are similar to those in Fig 4 C D and G representing FM sweep During one cycle of FM modulation the tone passes the unit s excitatory area twice In the case of AM sounds the unit is excited only once for every tone burst In order to facilitate the comparison of the response pattern to these two types of modulation the repetition rate in the case of AM sounds refers to pairs of sound bursts (cf Fig 1 B)

In order to ensure that the changes found in the response pattern with increasing sweep-rate were not influenced by the concomitant increase in the repetition rate of the modulation the slope of the trapezoidal modulation was varied and cor

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and falling (thin dashed lines tone sweep The vertical scale refers to the total spike counts For the spike counts in each peak multiplied by a factor of two in order to facilitate the comparison of the total spike counts and the number of spikes contained in each peak

F The relative height of the peaks in the histograms of the response to AM sounds as a function of repetition rate The different symbols indicate intensity of the tone bursts open circles 65 dB triangles 45 dB and closed circles 25 dB above threshold at the unit s CF

H The corresponding spike counts contained in the peaks in the histograms of the response to AM sounds The symbols correspond to those in graph F

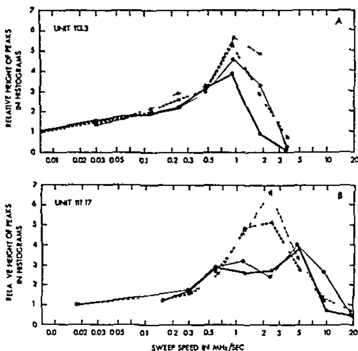


Fig 5 Relative height of the peaks in histograms of the responses to trapezoidally frequency modulated sounds where the shape of the modulation has been varied
A Heavy lines correspond to a case where the tone frequency changed during 60 % of the modulation cycle and thin lines indicate a modulation where the tone frequency changed during 15 % of the modulation cycle
B Heavy lines indicate the response to FM sounds where the tone frequency changed during 15 % and thin lines 7 5 % of the total modulation cycle
Graphs A and B represent the responses from two different units In both graphs solid lines represent the response to rising tone frequency sweeps and dashed lines to falling sweeps

respondingly the rate of tone frequency change at a certain sweep frequency The results of these experiments showed that the height of the peaks in the histograms in most units was related to the rate of change in tone frequency and was independent of the sweep repetition rate provided that the tone frequency passed over the unit s response area during a small fraction of the total modulation cycle Examples of the results of such experiments are seen in Fig 5 where the relative height of the peaks in the histograms of the response to trapezoidally modulated sounds for two different slopes is shown The height of the peaks in the histograms is plotted as a function of rate of change in tone frequency (in MHz/sec) The heavy lines and the thin lines in Fig 5 A represent a trapezoidal modulation where the tone frequency changed during 60 % and 15 % respectively of the modulation cycle Continuous lines represent rising frequency sweep and dashed lines falling sweep In Fig 5 B the corresponding symbols represent a modulation where the tone frequency changed during 15 % and 7 5 % of the modulation cycle The figures 5 A and B

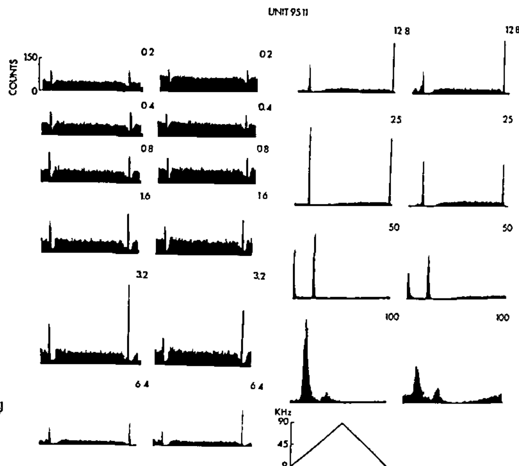


Fig 6 Cycle histograms of response to triangular modulated tones (25 dB above threshold at CF) of a unit with a CF of 19 8 kHz (columns 1 and 3) In columns 2 and 4 histograms of the responses to the same FM sound with a superimposed continuous tone whose frequency was equal to the unit's CF The intensity of the superimposed tone was 32 dB above *threshold at CF The sweep-rate (in sweeps/sec) is indicated by legend numbers*

are from two different units It can be seen that the different slopes of the trapezoidal modulation yield very similar results with respect to rate of change in tone frequency for rising tone frequency sweeps as well as for falling frequency sweeps This shows that the increase in height of the peaks in the histograms is primarily a function of the rate of change in tone frequency and only to a small extent influenced by the sweep frequency (modulation frequency) for constant sweep speed

In most of the units investigated the maximal value of the height in the histograms of the response to FM sounds increases somewhat with intensity as it does for the unit illustrated in Fig 4 In many units almost no maximum is seen near the threshold In some units the threshold to FM sounds was also investigated It was found that the threshold was independent of the rate of tone frequency

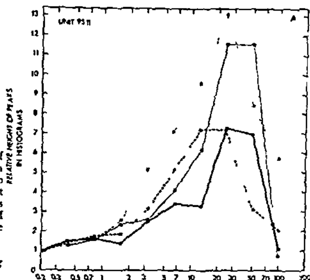


Fig 7 A The relative height of the peaks in the histograms shown in Fig 6 as a function of sweep frequency Heavy lines represent the response with a continuous tone superimposed on the FM tones and thin lines represent the response to FM tones alone Continuous lines Rising tone frequency dashed lines Falling tone frequency

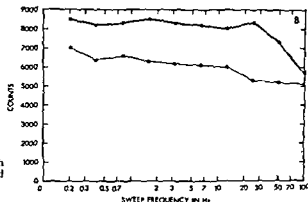


Fig 7 B Total spike counts during 1 min of stimulation with continuous tone superimposed (heavy line) and without a continuous tone (thin line)

change in the range studied although the peaks in the histograms of the responses to the same sounds above threshold showed a marked increase in height

In many units the maximum of the peaks in the histograms for falling frequency sweep is higher, and occurs at a somewhat slower sweep-rate than that for rising sweep This asymmetry becomes more apparent at higher sound intensities (*cf* Fig 4) In the range where the peak in the histograms for falling tone frequency is higher than the peak in the histograms for rising sweep the former is usually narrower than that for rising sweeps The resulting total number of discharges contained in the two peaks is usually almost the same The falling sweep thus gives rise to a somewhat more localized response than that of the rising frequency sweep

The rate of change in tone frequency which produced the highest histogram peaks varied from unit to unit For units with CFs in the range of 5 to 30 kHz the maxima occur at sweep-rates from 1 to 15 MHz/sec Units with a lower CF than 5

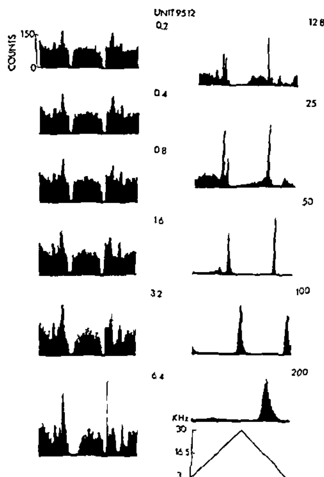


Fig 8 Cycle histograms of the response to frequency modulated tones of a unit with a weak excitatory response area to slowly varying tone frequency but with a pronounced area of suppression of spontaneous activity to tones with slowly varying frequency. Below the histograms is shown the shape of the frequency modulation

kHz usually have their maxima at somewhat lower sweep rate values but even units with a CF below 2 kHz can have a maximum above 1 MHz/sec. As mentioned above the values depend somewhat on the sound intensity (*cf* Fig 3 and 4). The sweep-rate at which the height of the peaks in the histograms has decreased to about the same value it possesses at low sweep-rates is usually more than twice the sweep rate where the maximum occurs

In order to see whether this sharpening of the response area to FM tones was influenced by the presence of another tone, experiments were performed in which a continuous tone with the same frequency as the unit's CF was superimposed on the FM tones. The results of such an experiment are depicted in Fig 6 which shows histograms of the response to FM tones with different sweep-rates when the continuous tone was superimposed. It is seen that at low sweep-rates there is a pronounced suppression of the activity on the high frequency side of the peaks. The height of the peaks in the histograms however, showed qualitatively the same relationship to the sweep-rate as those without the continuous tone but the maxi

mum in the height of the peaks was somewhat smaller with the t ne (see Fig 7) The unit s CF was 10 8 kHz the sweep tone was B threshold at CF and the continuous tone was 7 dB above the leve f th tone at the unit s CF

The majority of the units from which recordings were mad resp n d in very similar to those whose responses are shown in Fig 2 to 7 but a few differed strikingly in their response to FM sounds Some units show almo i excitatory area to tones with slowly varying frequencies but there was a marked suppression of their spontaneous activity in a certain range of tone frequenc F g 8 shows histograms of the responses of such a unit to sweep-tones At low s eep rates, almost no excitatory range is seen but a region of suppression is very pro nounced (The background activity which can be seen in th hi t grams of the response to tones with low sweep rates is spontaneous activity nd is thus n t due to any intentional stimulation) When the sweep-rate is increased the aspect f an excitatory area develops and becomes more pronounced At medium s eep rates the response pattern is not as regular as it is in ordinary units but at the highest sweep-rates used (50 to 200 Hz corresponding to 2 7 to 10 8 MHz/sec) the response pattern is very similar to that of an ordinary unit which has excitatory areas to tones with slowly varying tone frequency

Discussion

The response pattern to FM tones of all the units from which recordings were made changed as a function of sweep rate in such a way that the discharges became more and more concentrated near the unit s CF as the rate of tone frequency change was increased until a maximum was reached at a certain sweep-rate This response pattern was seen from sound levels of about 5 or 10 dB above the threshold and persisted almost unchanged throughout the investigated intensity range (to 70 dB above threshold) In some units the height of the peaks in the histograms of the responses to a falling tone frequency reached higher values than the responses to a rising sweep This difference was most pronounced at higher sound intensities

A series of experiments has failed to show any significant dependence of the unit s threshold on rate of tone frequency change for falling or rising frequency sweep in the range investigated *i e* from 0 1 to 100 Hz The height of the peaks in the histograms of the responses of units with CFs from 5 to 30 kHz often reached a maximum at sweep rates which correspond to a rate tone frequency change in the range of 15 MHz/sec The sweep-rate where the height of the peaks decayed to about the same value it had for low sweep-rates was often in the range of 50 MHz/sec

The periphery of the auditory system is usually compared to a spectral analyzer whose purpose it is to perform a spectral analysis of incoming sounds The charac teristic of each filter in such an anal zer determines the spectral and temporal resolution of the analyser The narrower the band width of the filters the higher the

spectral resolution Conversely, the broader the band width the higher the temporal resolution The upper limit of frequency change which can be handled faithfully by such filters is related to the square of the filter s bandwidth (measured for example, at the 3 dB points) According to Kupfmuller (1949), the frequency of the signal to be analyzed should not change faster than B^2/k Hz/sec, where B is the band width of the filter and k is a safety factor, usually chosen to be between 5 to 20 The response of a filter to a signal with a spectrum which varies at a rate above this limit is broadened and the amplitude of the response decreases The width of the tuning curve of rat cochlear nucleus units measured 10 dB above the threshold at CF is in the range of 1 5 kHz for units with CFs in the 10 kHz range (Moller 1970) The 3 dB band width is about half this value (*i e* approximately 750 Hz) Thus it had to be expected that the response to sweep-tones would already diminish at sweep-rates which are well below 0 5 MHz/sec The present study however shows that all the units from which recordings were made respond extremely well to tones whose frequency varies at a rate of 0 5 MHz/sec The histograms of the responses of most of the units from which recordings were made show in fact that the discharges are more localized around the unit s CF in response to tones with a more slowly varying frequency The units thus responded in an unexpected manner to tones with rapidly varying frequency on the basis of the characteristiscs of a linear filter with a band width equal to the width of the tuning curves

If a unit s excitatory area only is taken into consideration and the areas where a tone depresses the activity are disregarded a unit should respond to an FM sweep tone from the time it enters the unit s response area until it leaves it again The duration of the excitation thus becomes a function of the sweep-rate and the width of the unit s response area With that assumption pure tones within the unit s response area (*e g* at CF) delivered in bursts of proper duration would produce a similar response pattern to that produced by FM sweep-tones (In comparing the responses to FM and AM sounds it should also be taken into consideration that an FM sound does not produce a uniform excitation when it passes over a unit s response area but the shape of the excitation function might be somewhat rounded in accordance with the shape of the unit s response area The results of the present investigation however show that in most units the responses to short rectangular shaped tone bursts differ significantly from those to FM tones The reason for this may be that these units have their excitatory range surrounded by at least one area where a tone causes a suppression of the activity FM tones which sweep over a unit s response area from above its CF to below it will thus produce first a suppression followed by an excitation and thereafter possibly by another suppression

Sweep-tones will produce a di or triphasic excitation at the level of the cochlear nucleus (*cf* Suga 1965) compared to the short burst of a pure tone of constant frequency (at the unit s CF) which is assumed to produce a monophasic excitation Although the spectrum of a short tone burst narrows as time proceeds from the onset and the areas around the excitatory area may thus be more heavily excited

at the onset than they are later, this as far as frequency is concerned w l lways happen in a symmetrical fashion, whereas the FM sweep will acti ate of suppression and excitation in sequence

The results of the present investigation thus suggest that the pattern f incre d height in the histograms at a certain sweep-rate is mainly the result of the arrai e ment of the areas of suppression and excitation

The fact that a sharpening of the response is seen for falling as well as for rising frequency sweep makes it less conceivable that the sharpening sh uld have its origin in the excitation pattern on the basilar membrane as has been suggested by v Bergeijk (1964) He suggested that the basilar membrane might act in a similar way as the so-called collapsing filter used for pulse compression in a special type of radar (chirp radar) By delaying *e g* high frequencies more than low frequencies of a pulse of a FM signal whose frequency goes from high to low a compression of the pulse occurs as it passes through the filter If the direction of the sweep is reversed a concomitant expansion of the pulse occurs If the sharpening of the response to FM sounds had its origin in the vibration pattern of the basilar membrane the response to upgoing and downgoing FM sweeps would be completely different One could suggest that the slightly higher response to falling sweep seen in the present investigation could be due to the fact that most units have tuning curves which are steeper on the high frequency side than on the low frequency side When the tone frequency sweeps downwards it first hits the sharp edge of the excitatory response area when it sweeps upwards it hits a much less steep low frequency edge Whether this is the explanation however is questionable since such asymmetry of response with regard to sweep direction is also seen in those units which have symmetrical tuning curves It might be more conceivable that an asymmetry in the areas of suppression is responsible for this response pattern

Another explanation for this sharpening of the response to FM sounds might be found in the difference between the temporal integration which occurs in the response area where a tone causes excitation compared with that area where a tone causes a suppression of nerve activity Although this cannot be completely ruled out it seems less conceivable than the reasoning above The area of suppression both of spontaneous and driven activity which can be seen in the frequency range above the unit s response area is usually not significantly dependent on sweep-rate as is demonstrated in Fig 6 The suppression of the activity evoked by a tone of constant frequency equal to the unit s CF by a FM tone when its frequency passes over the region above the unit s CF is retained up to fairly high sweep rates (At sweep rates above 0 4 sweeps/sec an additional suppress on is seen immediately after the excitation This is assumed to be similar to the post excitatory suppression that al o is seen after stimulation with tone bursts) In only one unit could the suppression of spontaneous activity be seen to diminish at higher sweep-rates This unit however showed the same increase as other units in the height of the peaks in the histograms at a certain sweep-rate

In a recent study by Erulkar *et al* (1968) no systematic dependence has been

found between rate of tone frequency change and shape of the histograms of the responses of cochlear nucleus units It is uncertain whether the reason for this is that they used a rather narrow sweep range ($\pm 1/8$ octave) For a unit with CF around 13 kHz the maximal rate of frequency change used by Erulkar *et al* did not exceed 500 kHz/sec According to an earlier study (Moller 1969 b), and the results of the present paper, this rate of frequency change is usually below the range where the increase in peak height of the histograms is seen The narrow sweep-range used by Erulker *et al* may also have influenced the responses, since the tone probably did not go outside the unit's response range (*i e*, into the range of suppression) during the "dwell" periods at the two extreme frequencies

The fact that the present investigation shows no enhancement of the response to FM tones near threshold agrees with the findings of Suga (1964), who showed that units in the cochlear nucleus had the same threshold to FM tones, regardless of sweep direction The reason why Suga found a higher threshold for a 2 msec FM tone than a 2 msec steady tone at CF is probably due to the fact that the FM tone only stays within the unit's response area for a fraction of its length Since the response of these neurons represents the sound energy integrated over a (short) period of time the threshold of sounds shorter than this integration time depends on a sound's duration

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Action Potential Generation in Denervated Rat Skeletal Muscle

I. Quantitative Aspects

By

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Abstract

REDFERN, P. and S. THESLEFF. *Action potential generation in denervated rat skeletal muscle. I. Quantitative aspects.* Acta physiol. scand. 1971. *81.* 557—564.

Action potential generation was studied at various periods up to one week after denervation in individual muscle fibres of the extensor digitorum longus muscles of the adult rat. To allow a comparison of action potential generation at various stages of denervation, it was necessary to establish adequate conditions for spike generation. It was found that when fibres were locally polarized to a level of −90 to −100 mV, and the external calcium concentrations was increased to 4 mM, the peak rate of rise and the overshoot of the action potential were maximal. Between 30 and 40 hrs following section of the motor nerve, the mean maximal rate of rise of action potentials, recorded under the aforementioned conditions, was reduced by about one third, and remained at about this reduced level during the subsequent days. Two days after denervation the resting membrane potential was reduced from a mean of 82 mV in innervated muscle to a mean of 68 mV, and remained at about this level for the remaining 5 days studied. The electrical time constant and the input resistance of the muscle fibres gradually increased during the 7 days following denervation, the time constant by about 70 % and the input resistance by about 50 %. With anodal polarization in denervated muscle no significant correlation was found between the resting membrane potential and the maximal peak rate of rise of the spike. It was concluded that denervation produces a genuine reduction in the rate of rise of the action potential in muscle fibre.

Following denervation marked alterations occur in the electrophysiological properties of mammalian skeletal muscle. The main changes in the extensor digitorum longus (E.D.L.) muscle of the rat include a fall in the resting membrane potential and a gradual increase in membrane resistance and total capacitance (Albuquerque and Thesleff 1969, Albuquerque and McIsaac 1970). The electrical and chemical excitability of the muscle membrane is also changed, spontaneous action potentials appear and a gradual increase in the area of the membrane sensitive to acetylcholine is observed (Ginetsinsky and Shamarina 1942, Axelsson and Thesleff 1959, Miledi 1960).

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It has been shown (Albuquerque and Thesleff 1968) that action potential generation is impaired in muscles denervated 7—10 days, at which time the rate of rise of the action potential is reduced. It was not clear, however, whether the observed decrease in excitability was secondary to the reduction in the resting membrane potential, or attributable to other factors. The time course of the onset of the reduction in electrical excitability was also unknown and it was of interest to compare it with the appearance of the other changes in the membrane following denervation.

Experiments were therefore performed in which the resting membrane potential, the input resistance, the electrical time constant and action potential generation were measured in the E D L muscle of the rat at various periods following denervation. To reduce the complication of muscle fibre atrophy, the investigation was limited to the first seven days following denervation.

Methods

The E. D. L. muscle of male Wistar rats with a body weight of 180—220 g was denervated unilaterally under ether anesthesia close to the knee joint, about 5 mm from the entry of the nerve into the muscle. At various intervals following nerve section the denervated muscle and its contralateral innervated control were removed and mounted together in an organ bath. The bath fluid had the composition described by Liley (1956) except that the concentration of calcium was increased to 4 mM by the addition of calcium chloride (see results) and was bubbled with 5 % CO_2 in oxygen, giving the fluid a pH between 7.0 and 7.3. The temperature of the bath was maintained at 29° C ± 0.5° C. The membrane potential during rest and impulse activity was recorded from the surface fibres of the muscle using conventional glass capillary microelectrodes with a resistance of 4 to 10 Mohms; the input capacitance of the recording circuit with the microelectrodes was 5—10 pF.

To generate and record the action potential two microelectrodes were inserted into the fibre about 50 μm apart, one electrode, connected through a 100 Mohm resistance, was used to pass current and the other to record the potential change. Constant anodal current in steps of varying magnitude was passed through the membrane for 30 to 60 sec, a time found to give steady state conditions. This current terminated in a 5 msec cathodal shock adjusted in each case to produce an action potential with 1—3 msec latency. The technique of stimulation and recording generally produced little fall in resting potential and several spikes could be recorded from the same fibre. The rate of rise of the action potential was obtained by the use of a R. C. derivating circuit (100 pF, 100 Kohms).

The input resistance of single fibres was measured using pulses of varying intensity and 200 msec duration covering a range of anelectrotonic currents causing a potential change between 2 and 10 mV. The average result of such a series was used. The electrical time constant of the membrane was obtained by measuring the time required for the membrane potential change to reach 83 % of its plateau value (Boyd and Martin 1959).

A series of muscles were prepared by soaking the pair for 1 hour in a bath solution made hypertonic by the addition of 400 mM glycerol and then returning the muscles to isotonic solution. This procedure described by Howell and Jenden (1967) is known to disrupt the sarcotubular system in frog muscle (Eisenberg and Eisenberg 1968) and thereby to abolish excitation-contraction coupling (Gage and Eisenberg 1969).

Results

Resting membrane potential. On the second day following denervation the resting membrane potential was reduced from a mean of 82 mV to a mean of 68 mV and this value was little changed for the remaining 5 days of the period examined as shown in Fig. 1. The resting membrane potential appeared to fall rather abruptly, at about 30 hrs after denervation.

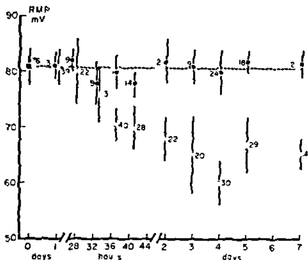


Fig 1 The effects of denervation on the resting membrane potential of individual fibres of the EDL muscle of the rat The open circles indicate the mean ± SD of values in denervated muscles, and the closed circles indicate the mean ± SD of values from the contralateral innervated muscles The abscissa shows the period of denervation on two different time scales The mean ± SD of the resting membrane potential of all innervated muscle fibres is indicated by the dotted line and values at zero time Each mean was obtained from at least two muscles and the value next to each point is the number of fibres examined

Effects of anodal polarization on action potential The marked difference in resting membrane potential between innervated muscle and muscle denervated for 2 or more days, made direct comparison of their action potentials meaningless, since spike generation is dependent on the level of polarization of the cell (Hodgkin and Huxley 1952) Anodal polarization restores the excitability of nerve and muscle depolarized by a variety of means (Frankenhaeuser and Hodgkin 1957) This technique was therefore adapted to the present study of action potential generation

Anodal currents allowed the membrane potential of the muscle cell to be locally set to values up to −120 mV and the relationship between this potential and the local generation of action current to be determined Responses were measured and expressed as the maximal rate of rise The maximum rate of rise reflects inward current at that moment and in the absence of voltage clamp conditions this is probably the most meaningful index of spike generation

Representative action potentials in an innervated and a 4 day denervated muscle at various levels of membrane polarization are shown in Fig 2 In both innervated and denervated muscle maximal peak rates of rise were observed when the membrane was polarized to −80 to −100 mV Further polarization failed to increase the rate of rise of the action potential and often reduced it At potential levels below −75 mV action current was greatly reduced (Fig 3) Glycerol treatment of muscle which abolished excitation contraction coupling, and allowed the recording of repeated spikes uncomplicated by mechanical response revealed the same essential relationship between the level of polarization and the maximal rate of rise of the spike showing that this relationship was not influenced by mechanical twitching (Fig 4)

Since peak responses were obtained at polarization levels of −80 to −100 mV all subsequent recordings of action potential in both control and denervated muscle were carried out at local potential between −90 and −100 mV

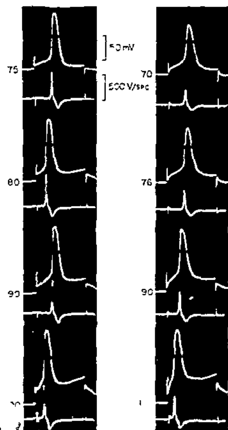


Fig 2

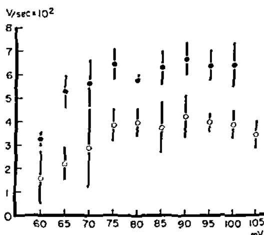


Fig 3

Fig 2 Consecutive action potentials (upper traces) and their first derivatives (lower traces) from an individual fibre of an innervated E D L muscle (left hand record) and from a fibre of a 4 day denervated E D L muscle (right hand record) The fibres were locally polarized to the potential levels indicated by the numbers and the gap in the recordings of the action potentials shows the zero potential of the cell The duration of the stimulating current pulse was 5 msec

Fig 3 The maximal rates of rise (ordinate) of action potentials at various levels of local membrane polarization (abscissa) in 4 day denervated muscles (open circles) and in the contralateral innervated muscles (closed circles) Each value is the mean ± S D of 5 to 16 measurements in two E D L muscles

External calcium is known to play an important part in membrane excitation The sodium activation *mechanism is in addition to membrane polarization determined* by the calcium ion concentration among other things (Frankenhaeuser and Hodgkin 1957) To establish the adequate concentration of calcium for action potential generation in both *innervated and denervated muscle experiments were made in* solutions containing 2, 4 6 and 8 mM calcium No significant differences in maximal rate of rise of the action potential was observed at these calcium concentrations, but the mean values indicated *that maximal rates of rise were obtained in the presence* of 4 mM calcium This concentration was therefore used in all experiments

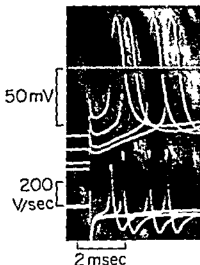


Fig 4 Consecutive and superimposed action potentials and their first derivatives from a single fibre of an innervated glycerol treated E D L muscle As shown by the record the level of membrane anodal polarization was changed between each action potential The broken line indicates the zero potential level of the fibre

Effects of denervation on action potential Having established the aforementioned conditions for spike generation in innervated muscle, it became possible to make a quantitative study of the effects of denervation on the action potential As shown in Fig 5 the average maximal rate of rise of the action potential was reduced on the second day following denervation from 630 V/sec to 370 V/sec. During the subsequent days the rate of rise of the spike remained at about this reduced level The amount by which the spike exceeded zero membrane potential *i e* overshoot was not as markedly changed by denervation A closer examination of the time course of the fall in the rate of rise of the action potential showed that it occurred quite rapidly between 30 and 40 hrs of sectioning the nerve

Denervation not only reduced the rate of rise of the action potential but also reduced its rate of repolarization and prolonged its duration (Fig 2) These changes were already apparent two days after denervation and were not markedly altered during the subsequent 5 days

The possibility that the fall in resting membrane potential was the direct cause of the observed reduction in action current was examined by analysing the data for muscles denervated for 2 or more days for a possible correlation between the resting membrane potential and the maximal rate of rise of the spike There was no significant relationship (correlation coefficient 0 056) Glycerol treatment of muscles is known to produce a variable fall in the resting membrane potential (Gage and Eisenberg 1969) and in our study the mean resting membrane potential of 136 innervated skeletal fibres following glycerol treatment was reduced to 63 ± 1 1 mV Among these glycerol treated muscle fibres there was also no significant correlation between the resting membrane potential and the maximal rate of rise of the action potential (correlation coefficient 0 008)

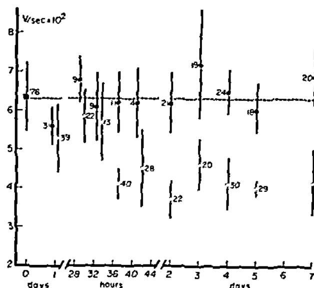


Fig 5 The maximal rate of rise of action potential (ordinate) in muscles denervated (open circles) for the time shown by the abscissa and in the contralateral innervated muscles (closed circles) Each value is the mean ± S D of measurements from at least 2 muscles and the adjacent figure indicates the number of fibres examined The mean ± S D for all the innervated muscles is indicated by the values at zero time and by the dotted line All recordings were made in fibres polarized to −90 to −100 mV

In the present recordings no effort was made to distinguish between end plate region and the rest of the fibre and therefore it is not possible to state whether the observed change in action potential generation developed initially in relation to the d plate region or simultaneously in the whole fibre

Effects of denervation on input resistance and electrical time constant The observed decrease in electrical excitability following denervation could reflect the lengthening of the electrical time constant observed in muscles following denervation (Albuquerque and Thesleff 1968) Table I shows the mean values of input resistance and electrical time constant of the fibres of innervated and denervated muscles Both properties increase gradually during the 7 days following denervation the time constant by about 70 % and the input resistance by about 50 % A similar gradual increase in these two parameters has been reported by Albuquerque and McIsaac (1970

TABLE I Influence of denervat n n mean S D membrane time constant and mean S D input resistance of single fibres f E D L muscle of the rat Figures within parenthesis are the number of fibres examined

Days of denervation	Membrane time constant msec	Input resistance Mohm
0	3 0 0 52 125	0 49 ± 0 127 (55)
2	2 7 0 73 19	
3	3 7 ± 0 37 2	0 50 ± 0 034 (10)
4	4 5 ± 0 55 21	—
5	—	0 66 0 142 (10)
6	5 2 ± 0 87 20	0 68 ± 0 115 (19
7	5 2 ± 0 46 19	0 72 ± 0 144 (22)

Discussion

In order to make a quantitative comparison of action potentials in different muscles it is necessary to establish either identical or optimal conditions for spike generation The observed fall in resting membrane potential following denervation precluded a direct comparison between the action potential of innervated and denervated muscle In the absence of adequate techniques for voltage clamping of muscle fibres it was decided to use anodal polarization With this procedure the membrane potential of the cell is locally changed at the site of the current electrode and from that point exponentially decaying over an area determined by the electrical length constant of the fibre which in both innervated and denervated E D L muscles is about 0 5 mm (Albuquerque and Thesleff 1968) Despite this limitation to the technique the fact that the action current as evidenced by the maximal rate of rise of the spike reached a plateau in both innervated and denervated muscles indicates that sufficient polarization levels had been reached A calcium concentration of 4 mM was found adequate for spike generation in innervated and denervated muscle

A comparison of the action potential under the aforementioned conditions showed that the rate of rise and the amount exceeding zero membrane potential were always reduced in muscle denervated for 2 or more days Since with the technique of anodal polarization no correlation was found between resting membrane potential and maximal rate of rise of the action potential in depolarized fibres it is unlikely that the fall in rate of rise following denervation was secondary to the reduction in resting membrane potential It can be concluded therefore that denervation produces a genuine reduction in the rate of rise of the action potential in the muscle fibre It is of interest that this reduction in spike generation and also the fall in resting membrane potential occurs abruptly at 30 or 40 hrs after denervation shortly after the time at which it is known that transmitter release from the degenerating nerve terminals stops Miledi and Slater 1968 Albuquerque and McIsaac 1970) It should also be mentioned that at this time the acetylcholine sensitive area of the muscle fibre membrane starts to spread

The fall in resting membrane potential and in the rate of rise of the action potential are the earliest post denervation changes in muscle Since these changes affect the entire muscle cell it seems reasonable to assume that they are the result of rapidly developing structural changes in the cell membrane

The reduction in the maximum rate of rise of the spike following denervation could be due to the observed lengthening of the electrical time constant However the change in the time constant following denervation was not coincident with the fall in the rise rate of the spike Another explanation would be that denervation reduced the number or the efficiency of the membrane sites for action potential generation The finding that the action potential following denervation becomes partly resistant to the blocking action of tetrodotoxin (Redfern, Lundh and Thesleff 1970) indicates that qualitative changes occur in the membrane sites responsible for spike generation

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Biochemistry of Catecholamine Storage: Some Similarities between Whole Sympathetic Nerve Trunk Vesicles and the Membranes of Adrenomedullary Vesicles

By

K B Helle, H Lagercrantz and L Stjar

It is well known that catecholamine (CA) storage vesicles of the ad   r   r   lla (= A vesicles) and sympathetic nerve trunk (= N vesicles) show a number   bio chemical similarities qualitatively The purpose of the present inv stigation has been to make the comparison between the two types of CA storing particles quantitative with respect to amounts of CA ATP, chromogranin A and lipids nd to dopamine β hydroxylase (DBH) activity

erve trunk   s d   rennial and 1970   I   grad ent f actions pH 6 0 and centrifuged for of solvent and centrifuged as before The pellet thus obtained was suspended in 1 % Triton X 100 and used as the source of water insoluble protein (WIS) The supernatants were pooled concentrated and assayed for soluble constituents

A vesicles were obtained from bovine adrenal glands and lysis was carried out in 5 mM   on of the protein was recovered   g av min The twice washed   ce of water in oluble protein

Protein was determined with the folin phenol reagent *Chromogranin A* was assayed im munologically by means of the double diffusion technique using agarose plates Helle 1970 *DBH* was assayed as described by Gibb Spector and Udenfriend 1967) using tyramine as the substrate The results have been expressed in units of DBH equal to 10^{-9} nmoles product formed after 10 min of incubation *Lipids* were analysed essentially as described by Blaschko *et al* (1967 *ATP* was measured by the firefly method *CA* were assayed fluorimetrically

Protein In agreement with earlier findings 80 % of the total protein of the A vesicles occurred in a water soluble (WS) form In the N vesicles on the other hand about 80 % was water insoluble

Chromogranin A By the double diffusion technique chromogranin A was detected in WIS fractions after solubilization in detergent and found to account for 21 % of the protein in the membranes of the A vesicles By the same method chromogranin A was found to represent 3 1 % of WIS protein of the N v (Table I)

TABLE I

Constituents	N vesicles				A vesicles			
	WIS I		III		WIS S4		WS SN1	
mg chromogranin A/mg protein	0.0082 ±0.0031	(4)*	0.031 ±0.010	(4)	0.21 ±0.05	(7)	0.27 ±0.11	(7)
DBH** units/mg protein	1.71 ±1.27	(3)	1.14 ±0.04	(3)	2.01 ±0.43	(7)	0.31 ±0.12	(7)
DBH units/mg chromogranin A	219		38		10.60		1.34	

* Values are expressed as means ± S.D., and the number of experiments are given in brackets.
** DBH units = 10^{-3} nmoles of product formed from tyramine after 10 min of incubation.

The results obtained for the splenic nerve preparations closely agree with those previously reported with similar methods (see Helle 1970). Thus the present results confirm that the WIS protein fractions of both the A and the N vesicle preparations contain considerable amounts of chromogranin A which apparently have escaped detection when the micro-complement fixation technique is used for the immunological assay: by the latter method only 0.002 and 0.04 mg chromogranin A could be detected per mg of protein in the particulate fractions of splenic nerve (De Potter et al. 1969) and A vesicle membranes (Winkler et al. 1970) respectively.

DBH activity was mainly confined to the WIS protein fraction of the A vesicles and the DBH/protein ratio of this fraction was comparable to that obtained for the WIS protein fraction of the N vesicles (Table I). It follows that the DBH/chromogranin A ratio for the A vesicles was significantly smaller than for the N vesicles. In the latter the microsomal band (F. I. Table I) showed a much higher ratio DBH/chromogranin A than the more dense N vesicles (F. III. Table I). This suggests that fraction F. I. in addition to microsomes may contain membranes of ruptured N vesicles, a different, less dense species of N vesicles or N vesicles in a different stage of development.

Lipids. Preliminary lipid analysis of the N vesicle preparations showed similarities in cholesterol and phospholipid content between the two types of storage vesicles with phosphatidylethanolamine and lecithin as the main phospholipides. In the chloroform methanol extract about 0.78 μmoles Pi/mg protein was found in the N vesicle preparation while Blaschko et al. (1967) found 2.83 μmoles Pi/mg N corresponding to about 0.45 μmoles Pi/mg protein in the A vesicle preparation. The A vesicles have been reported to have a relative high content of lysolecithin (Blaschko et al. 1967). Only trace amounts of this phospholipid could be detected in the N vesicles (Lagercrantz, to be published).

The molar NA/ATP ratio (NA = noradrenaline) in the purified N vesicles was found to be close to 4, as earlier observed in less pure preparations. During in-

cubation at 37° C for 15 min the amine/ATP ratio in N vesicles dropped to 1 4, while an essentially unchanged ratio was maintained in A vesicles, this is in agreement with earlier observations that ATP in the N but not in the A vesicles is retained upon amine release (Stjärne 1964)

The NA/protein ratio was 19 7 nmoles NA/mg protein The NA/cromogranin A ratio was 620 nmoles/mg (mean of four experiments range 310—1150) in keeping with previous reports (Helle 1970) This ratio is very similar to the CA-chromogranin ratio found in the extensively washed membranes of the A vesicles

Conclusion The chemical composition of the water insoluble matrix of the N and A vesicles seems to be closely similar with respect to lipid/protein and DBH/protein ratios The N and A vesicles appear to differ mainly in their content of water soluble constituents, *i e* CA, ATP and chromogranin A

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A Mucopolysaccharide-Protein Complex with Amine Binding Properties in Rat Thrombocytes

By

CARL HUGO ÅBORG and BÖRJE UVNÄS

From a series of observations in our laboratory on the uptake and release of histamine (Hi) and 5 hydroxytryptamine (5 HT) by rat mast cell granules it was concluded that these granules have binding properties similar to a weak cation exchange resin The amines were shown to be bound to carboxyl groups in the heparin protein complex which forms the bulk (> 95 %) of the granule matrix (Uvnäs Åborg and Bergendorff 1970) Suspension of amine containing granules in isotonic NaCl solution resulted in an instantaneous and complete depletion of the granule amine stores This was due to an equivalent exchange between sodium ions in the suspension medium and the amines at the cationic binding sites of the granules (Uvnäs *et al* 70 Bergendorff and Uvnäs 1971)

Thrombocyte granules also store Hi and 5 HT and in addition contain large quantities of adenosine triphosphate (ATP) This nucleotide is generally assumed to be essential for the storage of the amines the assumption being based on the fact that *in vitro* ATP forms complexes with both 5 HT (Da Prada and Pletscher 1968) and Hi (Schauer and Eder 1961)

The amine binding properties of the heparin protein complex of the mast cell granules led us to look for a similar complex in rat thrombocyte granules Such granules were obtained by sonication of thrombocyte rich plasma fractions suspended in isotonic sucrose After removal of coarse debris by centrifugation at $2000 \times g$ the supernatant was recentrifuged at $18\,000 \times g$ The sediment was resuspended in a small volume of isotonic sucrose and centrifuged through a 5 10 % Ficoll density gradient in isotonic sucrose at $18\,000 \times g$ Granule containing fractions were resuspended in small volumes of deionized water and centrifuged at $3000 \times g$ The resulting precipitate was washed again with deionized water and then either used as such or after drying *in vacuo* washing in chloroform methanol (to remove lipids) and drying All procedures were performed at or about 0 C

When the insoluble granule material was resuspended in aqueous solutions containing 5-HT, Hi or sodium ions (at pH 6 7 with admixture of ^{14}C-5 HT ^{14}C-Hi or ^{22}Na respectively cation uptake occurred Fig 1) With increasing concentrations of 5-HT or Na the uptake rose both uptake curves approaching the same maximal level—around 100 mueq mg granule material dry weight the histamine

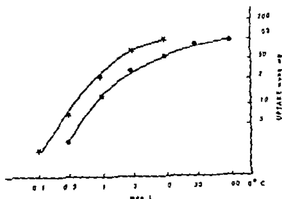


Fig 1 Uptake of 5 HT *** and sodium ●●● by the mucopolysaccharide-protein complex obtained from water lysed granules of sonicated rat thrombocytes Uptake calculated per mg complex dry weight and related to conc of 5 HT and Na^+ in deionized water
With rising 5 HT and sodium concentrations the uptake curves approach the same maximum

uptake curve was similar (For details of the experimental technique see corresponding experiments on isolated mast cell granules Uvnas *et al* 1970, p 7

The heparin of mast cell granules can be labelled *in vivo* with ^{35}S Since the amine binding material from the thrombocyte granules might also contain a mucopolysaccharide, attempts were made to label it with ^{35}S Rats were injected with $Na_2{}^{35}SO_4$ 5 mCi s c on each of two consecutive days and blood was drawn on the third day The thrombocytes were charged with ^3H 5 HT or ^3H Hi by incubating the rat plasma with ^3H 5 hydroxytryptamine or ^3H histamine (0 25 mCi) for 45 min at 37° C, and thrombocyte granules were then isolated as described above When centrifuged through the Ficoll gradient the distribution of the ^{35}S and the labelled amines coincided

On suspension of the amine binding granule material in 0 5 M NaCl solution centrifugation and subsequent passage of the supernatant through a Dowex 1 column the material separated into two fractions, a protein fraction in the void volume and a ^{35}S containing fraction eluted with 5 M HCl (Fig 2) Disc gel electrophoresis showed that the former fraction contained basic proteins The latter fraction contained hexosamine

Comments The results have demonstrated the presence in rat thrombocyte granules of a mucopolysaccharide protein complex capable of binding Hi 5 HT and Na Calculations showed that the binding capacity of this complex was sufficient to account for the storage of the quantities of 5 HT and Hi which normally occur in rat plasma Thus as previously found to be the case in mast cell granules the possibility exists that in the thrombocyte granules the biogenic amines are also stored in ionic linkage [illegible] as currently assumed [illegible] enhanced binding cap [illegible]

If our assumption that the thrombocyte amines are not stored in an amine-ATP complex is correct some other function has to be found, for the ample granule ATP stores

The amine storing granules in adrenals, as well as in peripheral and central

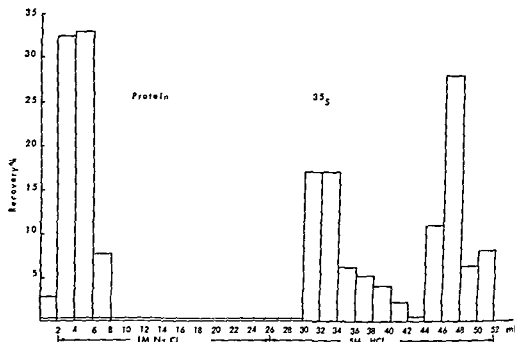


Fig 2 Separation on Dowex 1-X2 of protein and 35S (mucopolysaccharide) containing fractions from water lysed rat thrombocyte granules suspended in 0.6 M NaCl Rats previously injected with 2×0.5 mCi $Na_2{}^{35}SO_4$ (see text)

urones, also contain large quantities of ATP at present considered to be essential for the binding of the biogenic amines as ATP amine complexes In density gradient centrifugation studies of homogenates from various nervous tissue stores of biogenic amines from animals previously injected with $Na_2{}^{35}SO_4$ to label the mucopolysaccharides we have found a close correlation between amine- and 35S-containing fractions (Fillion Nosal and Uvnas to be published) We are presently trying to find further experimental support for our hypothesis that many biogenic amines are stored in simple ionic linkage to mucopolysaccharide protein complexes Such a mode of storage would allow an instantaneous release of the stored amines on exposure of the storage material to cations as has already been found to be the case for Hi and 5-HT in rat mast cell granules

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Recruitment in the Tonic Stretch Reflex

By

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In the tonic stretch reflex of the intercollicular decerebrate cat there is a linear increase in tension with increasing muscle length (Granit 1958, Matthews 1959) This increase in tension can to a large extent be accounted for by the inherent stiffness of the contracting muscle fibres even at lengths close to the maximal physiological extension of the muscle (Grillner and Udo 1971) In view of these findings there was reason to investigate the time course of the recruitment in the stretch reflex which can hardly be of the linear type (i e the same number of motor units should be recruited for each mm)

Intercollicularly decerebrate cats (n = 12) were used in which the soleus muscle was freed by removing the lateral gastrocnemius and the plantaris muscles and cutting the tendon of the denervated medial gastrocnemius The length of the soleus muscle was measured at minimal and maximal flexion in the ankle joint before removing the tendon with its bony insertion from the rest of the calcaneus The tendon and the distal half of the soleus muscle was dissected free from connective tissue The tendon was rigidly fixed with its bony insertion to a strain gauge (compliance 150 μ/kg) The distal and proximal parts of the tibia were rigidly fixed The muscle was extended at a low constant velocity (0 8 mm/sec) to the maximal length *in situ* Since the integrated EMG can hardly be regarded as strictly quantitative we have chosen to investigate the motor unit activity by recording single motor units that were sampled on the muscle surface with small spring mounted tungsten electrodes (resistance 100—200 kΩ) or glass microelectrodes (3 M NaCl resistance 0 5—2 MΩ) The motor unit activity was directly recorded on film and the discharge pattern was analyzed visually

Usually three such electrodes were used simultaneously for recording from different loci on the soleus muscle It was then often possible to record 3—5 recognizable motor units with large amplitude from each electrode Only motor units that were identified at maximal extension were accepted and then traced backwards' on the film to see at which muscle length and tension they were recruited It was carefully checked that units recorded in one lead were not also recorded in another Fig 1 A shows the number of single motor units recruited at various muscle tensions (len during the slow extension of the muscle up to the maximal muscle length I

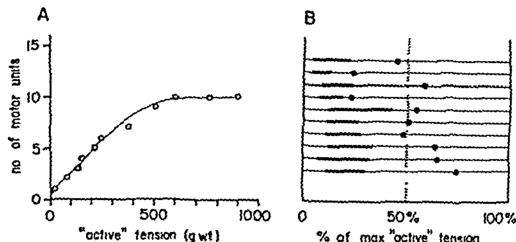


Fig Recruitment and tension in the tonic stretch reflex A shows the total number of active motor units as recorded simultaneously on the different locations (*cf* text) on the soleus muscle at various levels of active tension (*i e when the tension contributed by the inactive e g* denervated muscle has been subtracted *from the* total tension recorded) *during a slow stretch* at constant velocity Each horizontal line in *B* represents one stretch reflex the left edge of the bar *on each line indicates* at which *tension* 25 % of the motor units active at full extension have been recruited and the right edge when 75 % of the motor units were activated the dot to the right shows the point from which 100 % of the motor units were active The stretch reflexes had a final active tension above 600 g wt and showed the typical linear relation between length and tension The data are from 6 different cats and the total number of active *motor units that were included in one computation were always above 10*

ing that 9 out of 10 motor units were already recruited when the active tension had reached 50 % of its final value i e 920 g wt (observe that the passive tension contributed by the inactive muscle has been subtracted) Similar findings with a marked recruitment initially were always found when preparations with good stretch reflexes were used (>600 g wt active tension at maximal physiological extension)

Fig 1 B shows the relation between recruitment of motor units and the active tension developed in per cent of the maximal value of the stretch reflex for several preparations Each horizontal line represents one stretch reflex tested The left edge of the bar on each line shows the tension at which 25 % of the motor units active at the maximal physiological extension have been recruited The right edge of the bar indicates when 75 % recruitment is reached and the dot to the right shows the tension at which 100 % of the motor units recorded at those locations are active Also included in this graph are recordings from individual electrodes in which it was not possible to recognize individual motor units and follow them throughout the extension In these cases the total number of spikes per unit time in one lead was assumed to be proportional to the number of active motor units since virtually no frequency modulation occurs during slow extension at constant velocity (Grillner and Udo 1971) and since the firing rate of the soleus motor units under these conditions occurs in a rather narrow frequency band Grillner and Udo 1971) Fig 1 B

shows that recruitment of 75 % of the motor units regularly occurs at a tension much below 50 % of the maximal value

It can therefore be concluded that during constant velocity stretch there is in the tonic stretch reflex of the homogeneous soleus muscle in the intercollicularly decerebrate cat a comparatively large recruitment, which is reduced successively so that only a small fraction of the active motor units will be recruited at a length close to the maximal extension provided that there is a good stretch reflex It follows that the stiffness of the stretch reflex close to maximal extension is largely due to the stiffness of a fixed population of contracting muscle fibres (see also Grillner and Udo 1971), while at shorter muscle length recruitment of new motor units is an important factor Hence the recruitment of motor units in the tonic stretch reflex can be regarded as highly non linear

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Biphasic Mechanical Response of the Isolated Vas Deferens to Nerve Stimulation

By

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Isolated *in vitro* preparations of the vas deferens, stimulated via the hypogastric nerve (Huković 1961) or transmurally (Birmingham and Wilson 1963), have been extensively used as model systems for demonstration of adrenergic neurotransmission. Generally, the organ has been stimulated for shorter periods (2—10 sec) every one or two min. In the present studies on the isolated field stimulated rat vas deferens longer periods of stimulation, 30 sec every 60 or 90 sec, were used. It was noted that upon nerve stimulation, the initial, rapid contraction ("twitch") of the organ after about 5 sec was followed by a second, slower phase of contraction. Some observations on these two phases will be reported.

60 albino rats and 10 guinea pigs were used. The rat vas deferens was mounted in a 5 ml bath containing Tyrode solution at 37° C and stimulated transmurally at 12 V (biphasic pulses) 5 or 30 sec at 1 or 1.5 min interval, 1.0—1.5 msec duration and 4—25 stimuli/sec. The guinea pig vas deferens with the hypogastric nerve was mounted in a 50 ml bath and stimulated via the nerve using the same parameters as above (monophasic pulses). Contractions were recorded isotonically (load 0.2—0.5 g) on a Grass polygraph.

Results

1. After an equilibrium time of 5—90 min with repeated washing of the organ in the bath the "twitch" elicited by 5 sec stimulation was constantly followed by a slower contraction, generally with a greater final amplitude than the "twitch", when the stimulation period was extended to 30 sec (Fig. 1). The separation of the mechanical response in two phases was as a rule even more distinct in the guinea-pig preparation, stimulated via the hypogastric nerve (Fig. 2).

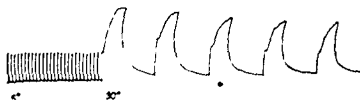


Fig. 1. Isolated rat vas deferens field stimulated for 5 or 30 sec at 1 min intervals with 12 V, 5 imp/sec, 1 msec duration.



g 2 Isolated guinea pig vas deferens stimulated as indicated by bars via the hypogastric rve for 30 sec at 1 min intervals with 12 V, 8 imp/sec 1 5 msec duration Phentolamine 10 g/ml guanethidine 4 μg/ml added to the bath at arrows

The 2 phases were present at all stimulation frequencies studied (4—25/sec)

After shifting from 5 to 30 sec stimulation the twitch of the second third and en fourth 30 sec contraction was often greater than the original ' twitch (Fig 1 id 3), but 5—10 min later the mechanical responses stabilized at a constant level hich could persist for at least 1 hr

α receptor blocking agents are in moderate concentrations known not to inhibit it rather to enhance the response of the vas deferens to nerve stimulation (Boyd hang and Rand 1960 and others) This effect on the twitch was confirmed in ie present study with *phentolamine* (0 5—10 μg/ml) The second phase of con action was however, immediately and constantly supressed in the rat vas deferens id almost totally disappeared in the guinea pig preparation (Fig 2)

β receptor blockers (*propranolol* or *dichloroisoproterenol* 1—10 μg/ml) in some ases slightly decreased the second phase but had no apparent effect on the twitch one of the phases was in any definite way influenced by *atropine* in concentrations f 0 1—100 μg/ml

Prostaglandins in very low concentrations have recently been found to depress he twitch of the isolated vas deferens (*cf* Euler and Hedqvist 1969 In ac ordance with this PGE_1 (1—5 ng/ml) in the present study markedly decreased or bolished the first phase of contraction of the guinea pig vas deferens while the econd phase was left relatively uninfluenced at the concentrations used (Fig 3)

Guanethidine (Fig 2) or *bretylium* 1—50 μg/ml profoundly depressed or com pletely abolished both phases



Fig 3 Isolated guinea p g vas deferens stimulated via the hypogastric nerve for 5 or 30 at 1 min intervals with 12 V 8 imp/sec 1.5 msec duration PGE_1 2 ng/ml at dot wa



Fig 4 Isolated vas deferens from a ra pretreated with reserpine (3 mg/k 48 hrs and 6 mg/kg 12 hrs before th expt) Field stimulated for 5 or 30 se at 1 min intervals 12 V, 5 imp/sec msec duration At dot washing

8 After *reserpine* pretreatment of rats (12 mg/kg 14 hrs or 3 mg/kg 48 hrs and 6 mg/kg 12 hrs before the expt) the noradrenaline content of the vas deferens decreased to very low values (0—77 ng totally, n = 6) but nevertheless the organ responded with "twitches" on stimulation for up to 10 hrs In no case was it possible to obtain a clearcut second phase of the reserpinized organs (Fig 4)

9 Vasa deferentia, postganglionically denervated by proximal vasotomy 1 week before the expt did not respond to transmural stimulation of less than 10 msec duration

The mechanisms behind the described biphasic mechanical response of the vas deferens i e the neurotransmitter release processes and the initiation of the contractile processes are at present obscure Even if the present results not necessarily require the assumption of two different transmitter processes in this organ this possibility cannot be excluded

In this connection it is of particular interest that several investigators have pointed out the possibility of a cholinergic mechanism in the neurotransmission of the vas deferens. Further pharmacological analysis of the described two phases of contraction might contribute to the elucidation of this possibility

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